THE MOLECULAR BASIS OF CANCER

MENDELSOHN
HOWLEY
ISRAEL
GRAY
THOMPSON

EDITION 4

ELSEVIER
SAUNDERS

Edition 4

The Molecular Basis of **Cancer**

John Mendelsohn, MD

Director, Khalifa Institute for Personalized Cancer Therapy
and Past President
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Joe W. Gray, PhD

Gordon Moore Endowed Chair
Chairman, Department of Biomedical Engineering
Director, OHSU Center for Spatial Systems Biomedicine
Associate Director for Translational Research
Knight Cancer Institute
Oregon Health and Science University (OHSU)
Portland, Oregon

Peter M. Howley, MD

Shattuck Professor of Pathological Anatomy
Department of Microbiology and Immunobiology
Division of Immunology
Harvard Medical School
Boston, Massachusetts

Mark A. Israel, MD

Director, Norris Cotton Cancer Center
Dartmouth-Hitchcock Medical Center
Lebanon, New Hampshire
Professor of Pediatrics and Genetics
Geisel School of Medicine at Dartmouth
Hanover, New Hampshire

Craig B. Thompson, MD

President and CEO, Memorial Sloan-Kettering Cancer Center
New York, New York

ELSEVIER
SAUNDERS

ELSEVIER
SAUNDERS

1600 John F. Kennedy Blvd.
Ste 1800
Philadelphia, PA 19103-2899

THE MOLECULAR BASIS OF CANCER **ISBN: 978-1-4557-4066-6**

Library of Congress Cataloging-in-Publication Data

The molecular basis of cancer / [edited by] John Mendelsohn, Peter M. Howley, Mark A. Israel, Joe W. Gray, Craig B. Thompson. -- 4.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-4557-4066-6 (hardcover : alk. paper)
I. Mendelsohn, John, 1936- editor of compilation. II. Howley, Peter M., editor of compilation. III. Israel, Mark A., editor of compilation. IV. Gray, Joe W., editor of compilation. V. Thompson, Craig (Craig B.), editor of compilation.
[DNLM: 1. Neoplasms--genetics. 2. Neoplasms--pathology. 3. Molecular Biology--methods. 4. Molecular Targeted Therapy. QZ 202]
RC268.5
616.99'4071--dc23 2013034109

Senior Content Strategist: Suzanne Toppy
Senior Content Development Specialist: Taylor Ball
Publishing Services Manager: Patricia Tannian
Senior Project Manager: Sharon Corell
Senior Book Designer: Louis Forgione

Printed in China

Last digit is the print number: 9 8 7 6 5 4 3 2 1

This book is dedicated to our wives:

Anne C. Mendelsohn
Jane E. Gray
Ann Howley
Susan J. Israel
Tullia Lindsten

Contributors

Stuart A. Aaronson, MD
Professor and Chair
Department of Oncological Sciences
Professor of Medicine
Mount Sinai School of Medicine
New York, New York

James L. Abbruzzese, MD
Chief
Division of Medical Oncology
Associate Director for Clinical Research
Duke Cancer Institute
Durham, North Carolina

Erika L. Abel, PhD
Lecturer/Advisor of Biology
Department of Biology
College of Arts and Sciences
Baylor University
Waco, Texas

Swarnali Acharyya, PhD
Research Fellow
Sloan-Kettering Institute
Memorial Sloan-Kettering Cancer Center
New York, New York

Bachir Alobeid, MD
Professor of Clinical Pathology and Cell Biology
Program Director, Hematopathology Fellowship Training Program
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Jennifer Amengual, MD
Assistant Professor of Medicine and Experimental Therapeutics
Center for Lymphoid Malignancies
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Kenneth C. Anderson, MD
Chief
Jerome Lipper Multiple Myeloma Center
Dana-Farber Cancer Institute
Boston, Massachusetts

Olena Barbash, PhD
Investigator
Oncology Research and Development
GlaxoSmithKline
Philadelphia, Pennsylvania

Robert C. Bast, Jr., MD
Vice President for Translational Research
Harry Carothers Wiess Distinguished University Chair for Cancer Research
Internist and Professor of Medicine
Departments of Experimental Therapeutics and Gynecologic Medical Oncology
Division of Cancer Medicine
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Stephen B. Baylin, MD

Professor of Oncology
Johns Hopkins University
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine
Baltimore, Maryland

Joseph R. Bertino, MD

Professor
Medicine and Pharmacology
University of Medicine and Dentistry of New Jersey
Robert Wood Johnson Medical School
Chief Scientific Officer
American Cancer Society Professor
Rutgers Cancer Institute of New Jersey
New Brunswick, New Jersey

Govind Bhagat, MD

Professor of Pathology and Cell Biology
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Mina J. Bissell, PhD

Distinguished Scientist
Life Sciences Division
Lawrence Berkeley National Laboratory
Berkeley, California

Scott A. Boerner, MS

Department of Internal Medicine
Wayne State University
Barbara Ann Karmanos Cancer Institute
Detroit, Michigan

Jessica E. Bolden, PhD

Gene Regulation Laboratory
Peter MacCallum Cancer Centre
Melbourne, Australia

Ernest C. Borden, MD

The Cleveland Clinic
Taussig Cancer Institute
Case Comprehensive Cancer Center
Cleveland, Ohio

Malcolm V. Brock, MD

Associate Professor of Surgery
Associate Professor of Oncology
Director of Clinical and Translational Research in Thoracic Surgery
Johns Hopkins University School of Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

Nelson E. Brown, MD, PhD

Instructor of Medicine, Molecular Oncology Research Institute
Tufts Medical Center
Boston, Massachusetts

Andrea Califano, Laureate in Physics

Clyde and Helen Wu Professor of Chemical Systems Biology
Columbia University
Director
JP Sulzberger Columbia Genome Center
Columbia University
New York, New York

George Adrian Calin, MD, PhD

Associate Professor
Department of Experimental Therapeutics
Division of Cancer Medicine
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Peter R. Carroll, MD

Professor and Chair
Department of Urology
Ken and Donna Derr-Chevron Distinguished Professor
Director of Clinical Services and Strategic Planning
University of California–San Francisco
Helen Diller Family Comprehensive Cancer Center
Associate Dean
University of California–San Francisco School of Medicine
San Francisco, California

Chakra Chennubhotla, PhD

Assistant Professor
Department of Computational and Systems Biology
University of Pittsburgh Drug Discovery Institute
University of Pittsburgh
Pittsburgh, Pennsylvania

Toni K. Choueiri, MD
Associate Professor of Medicine
Harvard Medical School
Attending Physician
Dana-Farber Cancer Institute
Boston, Massachusetts

Christopher L. Corless, MD, PhD
Professor of Pathology
Oregon Health and Science University
Chief Medical Officer
Knight Diagnostic Laboratories
Knight Cancer Institute
Portland, Oregon

Ana Luísa Correia, PhD
Life Sciences Division
Lawrence Berkeley National Laboratory
Berkeley, California

Rebecca Critchley-Thorne, PhD
Director
Biomarker and Diagnostic Development
Cernostics, Inc.
Pittsburgh, Pennsylvania

Carlo Maria Croce, MD
The John W. Wolfe Chair in Human Cancer Genetics
Chair
Department of Molecular Virology, Immunology, and Medical Genetics
Director
Human Cancer Genetics Program
The Ohio State University, Comprehensive Cancer Center
Columbus, Ohio

Alan D. D'Andrea, MD
Alvan T. and Viola D. Fuller American Cancer Society Professor
Harvard Medical School
Department of Radiation Oncology
Dana-Farber Cancer Institute
Boston, Massachusetts

Nancy E. Davidson, MD
Hillman Professor of Oncology
Director
University of Pittsburgh Cancer Institute and UPMC Cancer Center
Associate Vice Chancellor for Cancer Research
University of Pittsburgh
Pittsburgh, Pennsylvania

April Davis, BS
Research Technician
Comprehensive Cancer Center
Department of Internal Medicine
University of Michigan
Ann Arbor, Michigan

Changchun Deng, MD, PhD
Assistant Professor of Medicine and Experimental Therapeutics
Center for Lymphoid Malignancies
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

J. Alan Diehl, PhD
J. Samuel Staub, MD, Endowed Professor
Department of Cancer Biology
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

John DiGiovanni, PhD
Professor
Division of Pharmacology and Toxicology
College of Pharmacy
The University of Texas at Austin
Professor
Department of Nutritional Sciences
College of Natural Sciences, The University of Texas at Austin
Austin, Texas

Joseph Paul Eder, MD
Director of Experimental Therapeutics and the Phase I Research Group
Yale Cancer Center and Smilow Cancer Hospital
Yale University School of Medicine
New Haven, Connecticut

Suhendan Ekmekcioglu, PhD
Associate Professor
Department of Melanoma Medical Oncology
Division of Cancer Medicine
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Marc S. Ernstoff, MD
O. Ross McIntyre Professor of Medicine
Norris Cotton Cancer Center
Dartmouth-Hitchcock Medical Center
Lebanon, New Hampshire
Geisel School of Medicine at Dartmouth
Hanover, New Hampshire

James R. Faeder, PhD
Associate Professor
Department of Computational and Systems Biology
University of Pittsburgh Drug Discovery Institute
Pittsburgh, Pennsylvania

Eric R. Fearon, MD, PhD
Maisel Professor of Oncology
Division of Molecular Medicine and Genetics
Departments of Internal Medicine, Human Genetics, and Pathology
University of Michigan School of Medicine
Ann Arbor, Michigan

Lydia W. S. Finley, PhD
Postdoctoral Fellow
Cancer Biology and Genetics Program
Memorial Sloan-Kettering Cancer Center
New York, New York

Susan Fisher, PhD
Professor
Department of Obstetrics, Gynecology and Reproductive Sciences
Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research
University of California–San Francisco
San Francisco, California

Christopher D. M. Fletcher, MD, FRC Path
Professor
Department of Pathology
Harvard Medical School
Brigham and Women's Hospital
Boston, Massachusetts

Cyrus M. Ghajar, PhD
Life Sciences Division
Lawrence Berkeley National Laboratory
Berkeley, California

Adam B. Glick, PhD
Associate Professor
Center for Molecular Toxicology and Carcinogenesis
Department of Veterinary and Biomedical Sciences
The Pennsylvania State University
University Park, Pennsylvania

Albert Gough, PhD
Associate Professor
Department of Computational and Systems Biology
University of Pittsburgh Drug Discovery Institute
Pittsburgh, Pennsylvania

Ramaswamy Govindan, MD
Division of Oncology
Department of Medicine
Alvin J. Siteman Cancer Center
Washington University School of Medicine
St. Louis, Missouri

Jennifer R. Grandis, MD, FACS
Vice Chair for Research
Distinguished Professor of Otolaryngology and Pharmacology
University of Pittsburgh School of Medicine
Program Leader
Head and Neck Cancer Program
University of Pittsburgh Cancer Institute
Pittsburgh, Pennsylvania

Joe W. Gray, PhD
Gordon Moore Endowed Chair
Chairman
Department of Biomedical Engineering
Director
OHSU Center for Spatial Systems Biomedicine
Associate Director for Translational Research
Knight Cancer Institute
Oregon Health and Science University (OHSU)
Portland, Oregon

Douglas R. Green, PhD
Peter C. Doherty Endowed Chair of Immunology
St. Jude Children's Research Hospital
Memphis, Tennessee

Kirsten L. Greene, MD, MS
Associate Professor of Urology
Vice Chair, Education
Department of Urology
University of California–San Francisco
Helen Diller Family Comprehensive Cancer Center
Veterans Affairs Medical Center
San Francisco, California

Elizabeth A. Grimm, PhD
Professor
Department of Melanoma Medical Oncology
Deputy Head for Research Affairs
Division of Cancer Medicine
Frank McGraw Memorial Endowed Chair in Cancer Research
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Luca Grumolato, PhD
Professor
Chair of Excellence INSERM
Université de Rouen
Rouen, France
Instructor, Department of Oncological Sciences
Mount Sinai School of Medicine
New York, New York

Jian Gu, PhD
Assistant Professor
Department of Epidemiology
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

David A. Guertin, PhD
Assistant Professor
Program in Molecular Medicine
University of Massachusetts Medical Center
Worcester, Massachusetts

William C. Hahn, MD, PhD
Associate Professor of Medicine
Harvard Medical School
Chief
Division of Molecular and Cellular Oncology
Dana-Farber Cancer Institute
Senior Associate Member
Boston, Massachusetts
Cancer Program, Broad Institute of MIT and Harvard
Cambridge, Massachusetts

William N. Hait, MD, PhD
Global Head
Janssen Research and Development
Raritan, New Jersey

Matthew C. Havrda, PhD
Instructor in Pediatrics
Geisel School of Medicine at Dartmouth
Hanover, New Hampshire
Norris Cotton Cancer Center
Dartmouth-Hitchcock Medical Center
Lebanon, New Hampshire

Matthew L. Hedberg, MD/PhD Candidate
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania

Roy S. Herbst, MD, PhD
Professor of Medicine and Pharmacology
Chief of the Medical Oncology
Associate Director for Translational Science
Yale University School of Medicine
Yale Cancer Center
New Haven, Connecticut

Philip W. Hinds, PhD
Professor
Molecular Oncology Research Institute
Tufts Medical Center
Boston, Massachusetts

Daniela Hoehn, MD, PhD
Assistant Professor of Clinical Pathology and Cell Biology
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Peter M. Howley, MD
Shattuck Professor of Pathological Anatomy
Department of Microbiology and Immunobiology
Division of Immunology
Harvard Medical School
Boston, Massachusetts

Andrew C. Hsieh, MD
Helen Diller Family Comprehensive Cancer Center
University of California–San Francisco Medical Center
San Francisco, California

Patrick Hwu, MD
Department of Melanoma Medical Oncology
Center for Cancer Immunology Research
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Mark A. Israel, MD
Director
Norris Cotton Cancer Center
Dartmouth-Hitchcock Medical Center
Lebanon, New Hampshire
Professor of Pediatrics and Genetics
Geisel School of Medicine at Dartmouth
Hanover, New Hampshire

Tyler Jacks, PhD
Director
Koch Institute for Integrative Cancer Research
David H. Koch Professor of Biology
Investigator
Howard Hughes Medical Institute
Massachusetts Institute of Technology
Cambridge, Massachusetts

Johanna A. Joyce, PhD
Cancer Biology and Genetics Program
Memorial Sloan-Kettering Cancer Center
New York, New York

William G. Kaelin, Jr., MD
Professor of Medicine
Harvard Medical School
Dana-Farber Cancer Institute
Boston, Massachusetts

Hagop Kantarjian, MD
Department of Leukemia
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Brian Keith, PhD
Associate Investigator and Director of Education
Abramson Family Cancer Research Institute
Adjunct Professor
Department of Cancer Biology
University of Pennsylvania
Philadelphia, Pennsylvania

Ronan Kelly, MD, MBA
Assistant Professor of Oncology
Johns Hopkins University School of Medicine
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine
Baltimore, Maryland

Hasan Korkaya, PhD
Research Investigator
Comprehensive Cancer Center
Department of Internal Medicine
University of Michigan
Ann Arbor, Michigan

W. Michael Korn, MD
Associate Professor in Residence
Department of Medicine
(Gastroenterology and Medical Oncology)
University of California–San Francisco
San Francisco, California

Razelle Kurzrock, MD
Murray Professor of Medicine
Senior Deputy Center Director
Clinical Science Director
Center for Personalized Therapy and Clinical Trials Office
Vice Chair
Division of Hematology and Oncology
The University of California–San Diego
Moores Cancer Center
San Diego, California

Jill E. Larsen, PhD
Hamon Center for Therapeutic Oncology Research
Simmons Cancer Center
University of Texas Southwestern Medical Center
Dallas, Texas

Adrian V. Lee, PhD
Professor
Department of Pharmacology and Chemical Biology, and Department of Human Genetics
Director
Women's Cancer Research Center
University of Pittsburgh Cancer Institute
Magee-Womens Research Institute
University of Pittsburgh
Pittsburgh, Pennsylvania

J. Jack Lee, PhD
Professor
Department of Biostatistics
Division of Quantitative Sciences
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Anthony G. Letai, MD, PhD
Associate Professor in Medicine
Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts

Timothy Lezon, PhD
Assistant Professor
Department of Computational and Systems Biology
University of Pittsburgh Drug Discovery Institute
University of Pittsburgh
Pittsburgh, Pennsylvania

Long-Cheng Li, MD
Department of Urology
University of California–San Francisco
Helen Diller Family Comprehensive Cancer Center
University of California
San Francisco, California

Yan Li, PhD
Postdoctoral Fellow
Abramson Family Cancer Research Institute
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Scott M. Lippman, MD
Director
Moores Cancer Center
University of California–San Diego
San Diego, California

Yong-Jun Liu, MD, PhD
The Baylor Institute for Immunology Research
Baylor Research Institute
Dallas, Texas

Gregory Lizée, PhD
Associate Professor
Departments of Immunology and Melanoma Medical Oncology
Center for Cancer Immunology Research
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

A. Thomas Look, MD
Professor of Pediatrics
Harvard Medical School
Vice Chair for Research
Department of Pediatric Oncology
Dana-Farber Cancer Institute
Boston, Massachusetts

Patricia M. LoRusso, DO
Professor of Medicine, Director
Phase I Clinical Trials Program
Wayne State University
Barbara Ann Karmanos Cancer Institute
Detroit, Michigan

Scott W. Lowe, PhD
Chair
Geoffrey Beene Cancer Research Center
Cancer Biology and Genetics Program
Memorial Sloan-Kettering Cancer Center
New York, New York

David Malkin, MD
POGO Chair in Childhood Cancer Control
Medical Director
Pediatric Oncology Group of Ontario
Professor of Pediatrics
University of Toronto
Staff Oncologist
Division of Hematology/Oncology
Senior Scientist
Genetics and Genomic Biology Program
The Hospital for Sick Children
Toronto, Canada

Elaine R. Mardis, PhD
Co-Director
The Genome Institute
Professor of Genetics
Washington University School of Medicine
St. Louis, Missouri

Judith Margolin, MD
Associate Professor of Pediatric Hematology/Oncology
Texas Children's Cancer Center
Baylor College of Medicine
Houston, Texas

Joan Massagué, PhD
Chair
Cancer Biology and Genetics Program
Sloan-Kettering Institute
Memorial Sloan-Kettering Cancer Center
New York, New York

Lynn Matrisian, PhD
Vice President
Scientific and Medical Affairs
Pancreatic Cancer Action Network
Manhattan Beach, California

Frank McCormick, PhD, FRS
Director
Helen Diller Family Comprehensive Cancer Center
University of California–San Francisco
San Francisco, California

John Mendelsohn, MD
Director
Khalifa Institute for Personalized Cancer Therapy and Past President
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Gordon B. Mills, MD, PhD
Department Chair
Department of Systems Biology, Division of Cancer Medicine
Olga Keith Weiss Distinguished University Chair for Cancer Research
Professor of Medicine and Immunology
Department of Systems Biology
Division of Cancer Medicine
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

John D. Minna, MD
Hamon Center for Therapeutic Oncology Research
Simmons Cancer Center
Department of Pharmacology
University of Texas Southwestern Medical Center
Dallas, Texas

Mehdi Mollapour, PhD
Departments of Urology, Biochemistry, and Molecular Biology
SUNY Upstate Medical University
Syracuse, New York

Daniel Morgensztern, MD
Assistant Professor of Medical Oncology
Yale University School of Medicine
New Haven, Connecticut

Robert F. Murphy, PhD
Professor
Departments of Biological Sciences, Biomedical Engineering, and Machine Learning
Director
Ray and Stephanie Lane Center for Computational Biology
Carnegie Mellon University
Pittsburgh, Pennsylvania

Len Neckers, PhD
Urologic Oncology Branch
National Cancer Institute
Bethesda, Maryland

Owen A. O'Connor, MD, PhD
Professor of Medicine and Experimental Therapeutics
Center for Lymphoid Malignancies
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Peter H. O'Donnell, MD
Assistant Professor of Medicine
The University of Chicago
Chicago, Illinois

Steffi Oesterreich, PhD
Professor
Department of Pharmacology and Chemical Biology
University of Pittsburgh Cancer Institute
Magee-Womens Research Institute
University of Pittsburgh
Pittsburgh, Pennsylvania

Drew Pardoll, MD, PhD
Seraph Professorship in Oncology
Martin D. Abeloff Professorship
Co-Director
Cancer Immunology and Hematopoiesis Program
Professor of Oncology, Medicine, Pathology, and Molecular Biology and Genetics
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine
Baltimore, Maryland

Elspeth Payne, BSc, MB, ChB
Research Department of Haematology
School of Life and Medical Sciences
University College London Cancer Institute
London, United Kingdom

David G. Poplack, MD
Elise C. Young Professor of Pediatric Oncology
Head, Hematology/Oncology Section
Texas Children's Cancer Center and Hematology Service
Houston, Texas

Sean M. Post, PhD
Department of Leukemia
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Daniela F. Quail, MD
Cancer Biology and Genetics Program
Memorial Sloan-Kettering Cancer Center
New York, New York

Alfonso Quintás-Cardama, MD
Department of Leukemia
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Karen R. Rabin, MD, PhD
Assistant Professor of Pediatric Hematology/Oncology
Texas Children's Cancer Center
Baylor College of Medicine
Houston, Texas

Mark J. Ratain, MD
Leon O. Jacobson Professor of Medicine
The University of Chicago
Director
Center for Personalized Therapeutics
Associate Director for Clinical Sciences
Comprehensive Cancer Center
Chicago, Illinois

Julie D. R. Reimann, MD, PhD
Miraca Life Sciences
Tufts University School of Medicine
Newton, Massachusetts

Ignacio Romero, MD
Area Clinica Oncologia Ginecologica
Instituto Valenciano de Oncologia
Valencia, Spain

Eric Rubin, MD
Associate Director Clinical Science
The Cancer Institute of New Jersey
Professor of Medicine and Pharmacology
University of Medicine and Dentistry of New Jersey
Robert Wood Johnson Medical School
New Brunswick, New Jersey

Davide Ruggero, PhD
Associate Professor of Urology
Helen Diller Family Comprehensive Cancer Center
University of California–San Francisco
San Francisco, California

David M. Sabatini, MD, PhD
Professor of Biology
Whitehead Institute for Biomedical Research
Koch Center for Integrative Cancer Research at MIT
Investigator
Howard Hughes Medical Institute
Cambridge, Massachusetts

Ahmed Sawas, MD
Instructor of Clinical Medicine
Center for Lymphoid Malignancies
Department of Medicine
Division of Hematopathology
Department of Pathology and Cell Biology
Columbia University Medical Center–College of Physicians and Surgeons
The New York Presbyterian Hospital
New York, New York

Eric S. Schafer, MD, MHS
Assistant Professor of Pediatric Hematology/Oncology
Texas Children's Cancer Center
Baylor College of Medicine
Houston, Texas

Rachna T. Schroff, MD
Assistant Professor
Department of Gastrointestinal Medical Oncology
Division of Cancer Medicine
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Steven I. Sherman, MD
Professor and Chair
Naguib Samaan Distinguished Professor of Endocrinology
Department of Endocrine Neoplasia and Hormonal Disorders
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Sabina Signoretti, MD
Associate Professor of Pathology
Harvard Medical School
Associate Pathologist
Brigham and Women's Hospital
Dana-Farber Cancer Institute
Boston, Massachusetts

Branimir I. Sikic, MD
Professor of Medicine (Oncology)
Director
Stanford Clinical and Translational Research Unit
Co-director
Stanford University Center for Clinical and Translational Education and Research (Spectrum)
Stanford, California

M. Celeste Simon, PhD
Investigator
Howard Hughes Medical Institute
Professor
Department of Cell and Developmental Biology and Abramson Family Cancer Research Institute
University of Pennsylvania
Philadelphia, Pennsylvania

Paul T. Spellman, PhD
Associate Professor
Department of Medical and Molecular Genetics
Knight Cancer Institute
Oregon Health and Science University
Portland, Oregon

Josh Stuart, PhD
Associate Professor
Biomolecular Engineering Department
Associate Director
Center for Biomolecular Science and Engineering
Baskin School of Engineering
University of California–Santa Cruz
Santa Cruz, California

Rishi Surana, MD/PhD Candidate
Tumor Biology Training Program
Lombardi Comprehensive Cancer Center
Georgetown University
Washington, District of Columbia

Tanja Meyer Tamguney, PhD
Director of Research
SillaJen
Postdoctoral Scholar–McCormick Laboratory
Helen Diller Family Comprehensive Cancer Center
University of California–San Francisco
San Francisco, California

D. Lansing Taylor, PhD
Professor of Computational and Systems Biology
Director
University of Pittsburgh Drug Discovery Institute
University of Pittsburgh Cancer Institute
Pittsburgh, Philadelphia

Mario D. Terán, MD
Postdoctoral Fellow
Johns Hopkins University School of Medicine
Division of Thoracic Surgery
Baltimore, Maryland

Craig B. Thompson, MD
President and CEO
Memorial Sloan-Kettering Cancer Center
New York, New York

Giovanni Tonon, MD, PhD
Head
Functional Genomics of Cancer Unit
Division of Molecular Oncology
San Raffaele Scientific Institute
Milan, Italy

Robert A. Weinberg, PhD
Daniel K. Ludwig Professor for Cancer Research
Whitehead Institute for Biomedical Research
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts

Louis M. Weiner, MD
Director
Lombardi Comprehensive Cancer Center
Professor
Department of Oncology
Francis L. and Charlotte G. Gragnani Chair
Department of Oncology
Georgetown University
Washington, District of Columbia

Danny R. Welch, PhD
Professor and Chair
Adjunct Faculty of Department of Molecular and Integrative Physiology
The University of Kansas Medical Center
Kansas City, Kansas

Eileen White, PhD
Associate Director for Basic Science
Rutgers Cancer Institute of New Jersey
Professor, Molecular Biology and Biochemistry
Rutgers University
New Brunswick, New Jersey

Max S. Wicha, MD
Professor
Department of Internal Medicine
Director
University of Michigan Comprehensive Cancer Center
Distinguished Professor of Oncology
University of Michigan
Ann Arbor, Michigan

Monte M. Winslow, PhD
Assistant Professor
Department of Genetics
Stanford University School of Medicine
Stanford, California

H. Ewa Witkowska, PhD
Adjunct Professor
Department of Obstetrics, Gynecology, and Reproductive Sciences
University of California–San Francisco
San Francisco, California

Xifeng Wu, MD, PhD
Professor and Chair
Department of Epidemiology
The University of Texas M. D. Anderson Cancer Center
Houston, Texas

Stuart H. Yuspa, MD
Head
In Vitro Pathogenesis Section
Laboratory Co-Chief
Laboratory of Cancer Biology and Genetics
Center for Cancer Research
Bethesda, Maryland

Preface

Research in molecular genetics and cancer biology and advances in analytical technologies have revolutionized our understanding of cancer. Over the past three decades, there has been a massive acceleration in discoveries and observations that explains the genetic basis of cancer, a disease that until recently was thought about primarily in purely descriptive terms. Conversely, the study of malignancy has transformed our understanding of the molecular and genetic processes that govern the growth and proliferation of normal cells.

By 1995, our knowledge had expanded to the point that we felt it worthwhile to write a textbook describing *the molecular basis of cancer* for students, researchers, and providers of clinical care from a variety of disciplines. The aim in this fourth edition of the textbook continues to explain, rather than to merely recount.

Five editors, selected for their diverse expertise and their reputations as educators, met to design a sequence of sections and chapters that would lead the reader from the basic genetic and molecular mechanisms of carcinogenesis, to the molecular and biological features of cancer cell growth and metastasis, then to advances in sequencing technologies and bioinformatics that enable personalized risk assessment and diagnostics, followed by a description of molecular and genetic abnormalities that drive the common types of cancer, and finally to the molecular basis for new, targeted approaches to cancer therapy.

A purpose of this textbook is to describe the scientific underpinnings that will enable clinicians and other professionals who manage cancer patients to better understand the disease and its therapy. This book will be of equal, or possibly greater, interest to laboratory and clinical investigators in biomedical research and to advanced students and trainees, who need to understand the molecular mechanisms that govern the functioning and malfunctioning of malignant cells. Although the chapters follow a sequence that moves from pathogenesis to therapy, each chapter stands alone in its treatment of the subject matter.

Cancer arises as a result of genetic and epigenetic alterations that either enhance or diminish the activities of critical pathways that mediate normal cellular activities. Impaired capacity to repair genetic alterations can contribute to the likelihood that cells accumulate these genetic abnormalities, leading to malignant transformation. The disease is not merely a disorder of individual transformed cells. These cells grow into tumor masses and attract a blood supply, and they invade through surrounding tissues and metastasize. Molecular influences from the environment around the cancer cells contribute importantly to the capacity of genetically altered cells to produce malignant tumors.

A remarkable lesson gained from cancer research is that the strategies utilized by widely divergent cell lineages to regulate growth and differentiation share common molecular pathways. The accumulation of mutations and altered expression of genes critical for these pathways is a recurrent theme observed in many different types of cancer. Cancers also appear to select for genetic abnormalities that may be most advantageous for escape from normal regulatory mechanisms in their particular microenvironments.

What is most exciting today is the active dialogue between clinical investigators and laboratory scientists who share an interest in applying the new knowledge of genetics and molecular biology to the early diagnosis, targeted treatment, and improved prevention of disease. Today we have the opportunity to select treatments for clinical administration from among hundreds of new biological and chemical anticancer agents targeting pathways altered by specific molecular irregularities that result from aberrant genes. It is only recently that we can detect the genetic aberrations in cancer specimens from individual patients in a reasonable time frame and at a reasonable cost. This means that genomic assays can be used to select therapies that target the products of the aberrant genes in a patient's cancer and are more likely to provide benefit for that patient. The knowledge we present in this textbook should supply a basis upon which these new approaches to cancer therapy can be evaluated and implemented by those interested in understanding and critically assessing the many new products of the biotechnology revolution.

The editors are delighted that we were able to recruit as contributing authors outstanding investigators who are excited about the challenge of presenting their areas of expertise in a textbook format. In many cases this has required more time and effort than they initially anticipated, and we are grateful for their dedication. We hope that we have come at least part of the way toward achieving what we set out to do. We have been assisted and encouraged by the professionals at Elsevier, as well as the patient and ever-essential help of the secretaries in our offices.

John Mendelsohn, MD
Joe W. Gray, PhD
Peter M. Howley, MD
Mark A. Israel, MD
Craig B. Thompson, MD

William C. Hahn and Robert A. Weinberg

1 Cancer: A Genetic Disorder

Our understanding of the origins of cancer has changed dramatically over the past three decades, due in large part to the revolution in molecular biology that has altered the face of all biomedical research. Powerful experimental tools have been thrust into the hands of cancer biologists. These tools, including newly devised and implemented technologies that permit the interrogation of entire genomes, have made it possible to uncover and dissect the complex molecular machinery operating inside the single cell, normal and malignant, to understand its operations, and to pinpoint the defects that cause cancer cells to proliferate abnormally.

Three decades ago, at least three rival models of cancer's origins had substantial following among those interested in the roots of cancer. One model portrayed cancer as a disease of abnormal differentiation. According to this thinking, the changes in cell behavior that occur during the process of development run awry during tumor progression, causing cells to make inappropriate choices in moving up or down differentiation pathways. This concept of cancer's origins had important implications for the molecular origins of cancer: because the process of differentiation involves changes in cell phenotype without underlying changes in the genome, this model suggested that cancer was essentially an epigenetic process—a change in cell behavior without an underlying change in its genetic constitution.

An alternative model was advanced by the virologists. By the early 1970s, a number of distinct cancer-causing viruses had been catalogued in various animal species and in humans. These ranged from the Rous sarcoma virus, whose discovery reached back to the first decade of the century, to Shope papillomavirus, Epstein-Barr virus, papovaviruses such as SV40 and polyomavirus, and a variety of retroviruses that infected various mammals and birds. The existence of these viruses suggested that similar agents operated to trigger human tumors. Such hypothetical human tumor viruses were thought capable of insinuating themselves into human cells and transforming them from a normal to a malignant growth state.[1]

Yet another way of explaining cancer's origins was advanced by those who were impressed by the increasing connections being forged between carcinogens and mutagens. More than half a century of experiments had demonstrated the abilities of radiation as well as a vast array of chemicals to induce tumors in animals and occasionally in humans. Independent of this research, *Drosophila* and bacterial geneticists had documented the abilities of some of these carcinogenic agents to act as mutagens. The most influential of these experiments was to come from the laboratory of Bruce Ames. In the mid-1970s, Ames described a correlation between the mutagenic potencies of various chemical compounds and their respective potencies to induce tumors in laboratory animals.[2]

Ames' correlation (Figure 1-1) yielded the inference that the carcinogenic powers of agents derive directly from their abilities to damage genes and thus the DNA of cells. This strengthened the convictions of those who had long embraced the notion that cancer cells were really mutants and that their abnormal behavior derived from mutant genes that they carried in their genomes. This model implied that such mutant genes arose through somatic mutations, i.e., mutations that occur in somatic tissues during the lifetime of an organism and alter genes that were pristine at the moment of conception.

This last model of cancer's origins would eventually dominate thinking; the other two models largely fell by the scientific wayside. As the 1970s progressed, the search for tumorigenic viruses associated with most types of common human cancers bogged down. Human papillomavirus (HPV) clearly had strong associations with cervical carcinomas, Epstein-Barr virus (EBV) with Burkitt's lymphomas in Africa and nasopharyngeal carcinomas in southeast Asia, and hepatitis B and C viruses (HBV, HCV) with hepatocellular carcinomas in east Asia. Together, these accounted for as much as 20% of tumors worldwide.[3] However, the remaining types of cancers, and thus the vast majority of human cancers arising in the Western world, had no obvious viral associations in spite of extensive attempts to uncover them.

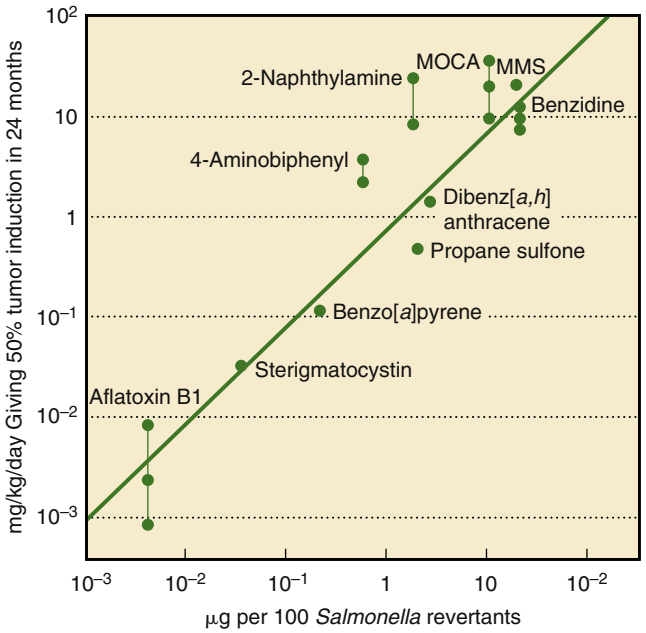


Figure 1-1 Relationship of carcinogenic to mutagenic potencies of chemical compounds The ability to quantify both the mutagenic potencies of a variety of chemical compounds, measured in the Ames mutagenesis test, and to relate this to their carcinogenic potencies, as measured in laboratory rodents, allowed this graph and correlation to be made between the two mechanisms of action. *(Adapted from Meselson M et al. In: Hiatt HH et al., eds.,* Origins of Human Cancer, Book C: Human Risk Assessment. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1977.)*

The epigenetic model of cancer lost its attractiveness largely because an extensive array of mutant growth-controlling genes was discovered in the genomes of human tumor cells. So the focus shifted increasingly to genes, more specifically the genomes of cancer cells. Cancer genetics in the 1970s and early 1980s became a branch of somatic cell genetics—the genetics of cells and their somatically mutated genes. Indeed, advances in the technology of DNA sequencing have now enabled the enumeration of mutations present in specific cancer genomes and will eventually lead to a compendium of recurrent genetic alterations in human cancers.

The Discovery of Cellular Oncogenes

The notion that cancer cells were mutants should have motivated a systematic search for genes that suffered mutation during the development of tumors. Moreover, these mutant genes should possess another property: they needed to specify some of the aberrant phenotypes ascribed to tumor cells, including alterations in cell shape, decreased dependence on external mitogenic stimuli, and an ability to grow without tethering to a solid substrate (anchorage independence). The fact that viruses were not important causative agents of most types of human tumors generated another conclusion about these cancer-causing genes: they were likely to be endogenous to the cell rather than being imported into the cell from some external source. Stated differently, it seemed likely that these cancer genes were mutant versions of preexisting normal cellular genes.

In the 1970s, when this line of thinking matured, the experimental opportunities to test its validity were limited. The human genome, which harbored these hypothetical cancer genes, represented daunting complexity. Its vastness precluded any simple, systematic survey strategy designed to locate mutant growth-controlling genes within cancer cells. Indeed, it is only now, three decades later, that the means, deep sequencing of cancer genomes, for conducting effective systematic surveys for cancer genes has been developed. Thus the discovery of cancer-causing genes—oncogenes as they came to be called—depended on a circuitous, indirect experimental strategy.

Ironically, it was tumor viruses, in the midst of being discredited as important etiologic agents of human cancer, that led the way to finding the elusive cancer genes. Varmus and Bishop's study of the Rous sarcoma virus (RSV) broke open the puzzle. Their initial agenda was to understand the replication strategy of this chicken virus. However, in the years after 1974, they focused their attentions to unraveling the mechanism used by RSV to transform an infected normal cell into a tumor cell.

Earlier work of others had indicated that a single gene, named *src*, carried the vital cancer-causing information present in the viral genome. Accordingly, the Varmus and Bishop laboratory launched a research program to trace the origins of this virus-associated *src* oncogene. In fact, the origins of most viral genes were obscure, shrouded in the deep evolutionary past. It seemed that most viruses and thus their genes originated hundreds of millions of years ago, perhaps as derivatives of the cells that they learned to parasitize.

However, as this team reported in 1976, the *src* gene behaved differently: it was a recent acquisition by the Rous virus. Many closely related retroviruses shared with RSV an ability to replicate in chicken cells and a very similar set of genes needed for viral replication. However, these other viruses lacked the *src* gene and the ability to transform infected cells into cancer cells, suggesting that the *src* oncogene carried by RSV was a relatively recent genetic acquisition. The Varmus-Bishop group soon traced the origins of the *src* gene to an unexpected source—a closely related gene that resided in the genome of normal chickens and, by extension, in the genomes of all vertebrates. They named this gene *c-src* (cellular *src*) to distinguish it from the *v-src* (viral *src*) oncogene carried by the virus.[4]

The Varmus-Bishop evidence converged on a simple conceptual model. It explained all their observations and ultimately much more. The progenitor of RSV lacked the

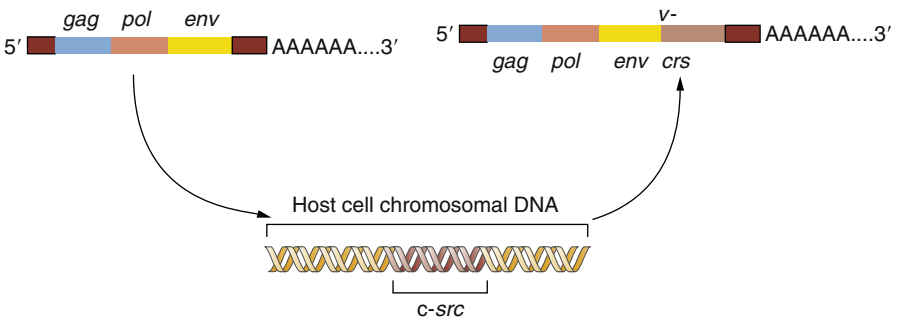


Figure 1-2 The origin of the Rous sarcoma virus *src* oncogene The acquisition of the v-*src* oncogene by a precursor of Rous sarcoma virus apparently occurred when an avian leukosis virus (ALV) lacking this oncogene infected a chicken cell and appropriated the cellular c-*src* proto-oncogene, thereafter carrying this acquired gene and exploiting it to transform subsequently infected cells.

v-*src* gene but grew well in chicken cells. During one of its periodic forays into a chicken cell, this ancestor virus picked up a copy of the c-*src* gene and incorporated it into its own viral genome. Once *src* was present within the viral genome, this slightly remodeled gene—now v-*src*—was exploited by RSV to transform cells it encountered in subsequent rounds of infection.

This provided a testimonial to the cleverness and plasticity of retroviruses, which seemed able to capture and then exploit normal cellular genes to do their bidding. But another implication was even more important: the Varmus-Bishop work pointed to the existence of a normal cellular gene, the c-*src* gene, that seemed to possess a latent ability to induce cancer. This cancer-causing ability was unmasked when the c-*src* gene was abducted by the chicken retrovirus that became the progenitor of RSV (Figure 1-2).

The c-*src* gene was named a *proto-oncogene* to indicate its inherent potential to become activated into a cancer-causing oncogene. Within several years, it became clear that as many as a dozen other tumorigenic retroviruses also carried oncogenes, each of which had been abstracted from the genome of an infected vertebrate cell.[5,6] Hence, there were many proto-oncogenes in the normal cell genome, not just c-*src*. Each seemed to be present in the DNA of a normal mammalian or avian host species, and by extension, present as well in the genomes of all vertebrates.

These discoveries were momentous because they demonstrated that normal cellular genes had the ability to induce cancer if removed from their normal chromosomal context and placed under the control of one or another retrovirus. Still, a key piece was missing from this puzzle. Retroviruses seemed to be absent from most, indeed from almost all, human tumors. Could proto-oncogenes ever become activated without direct intervention by a marauding retrovirus?

An obvious response was that proto-oncogenes might be altered by mutational events that did not remove these genes from their normal chromosomal roosts. Instead, these mutations would alter proto-oncogenes in situ in the chromosome by affecting either the control sequences or the protein-encoding sequences of these genes. This notion led to another question: If some proto-oncogenes could become activated by somatic mutations, such as those inflicted by chemical or physical carcinogens, would these be the same proto-oncogenes that were the targets of mobilization and activation by retroviruses?

In 1979 and 1980, answers came, once again from unexpected quarters. These newer experiments depended on the use of gene transfer, also known as *transfection*. The transfection procedure could be used to convey DNA, and thus genes, from tumor cells into normal recipient cells. The goal here was to see whether the transferred tumor cell DNA could induce some type of malignant transformation in the recipient cells. Success in such an experiment would indicate that the transferred gene(s) previously operated in the donor tumor cell to induce its transformation.

These transfection experiments succeeded (Figure 1-3). DNA extracted from chemically transformed mouse fibroblasts was able to induce normal mouse fibroblasts to undergo transformation.[7] Retroviruses were clearly absent from both the donor tumor cells and the recipients that underwent transformation and so could not be invoked to explain the cancer-causing powers of the transferred DNA. Soon the identity of these transferred genes, which functioned as oncogenes, became apparent. They were members of the *ras* family of oncogenes, which had initially been discovered through their association with rodent sarcoma viruses.[5,8] These rodent retroviruses had acquired *ras* proto-oncogenes from normal rodent cells, much like RSV, which had stolen a copy of the *src* proto-oncogene from a chicken cell.

Unanswered by this was the genetic mechanism that imparted oncogenic powers to the tumor-associated *ras* oncogene, more specifically an H-*ras* oncogene. It soon became clear that the tumor-associated H-*ras* oncogene was closely related to, indeed virtually indistinguishable from, a normal H-*ras* proto-oncogene that was present in the genomes of all vertebrates. Still, the tumor-associated *ras* oncogene carried different information than did the precursor proto-oncogene: the oncogene caused the malignant transformation of cells into which it was introduced, whereas the counterpart proto-oncogene had no obvious effects on cell phenotype. This particular puzzle was solved in 1982 with the finding that an H-*ras* oncogene cloned from a human bladder

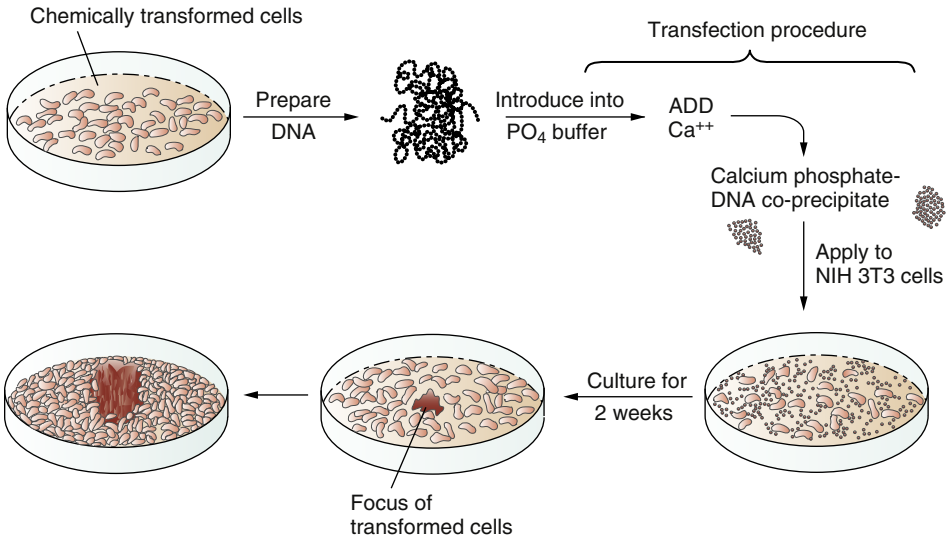


Figure 1-3 Transfection of a cellular oncogene The fact that the carcinogenicity of various chemical compounds was correlated with their mutagenicity suggested that cancer cells often carry mutant, cancer-inducing genes, i.e., oncogenes, in their genomes. This could be proven by an experiment in which DNA was extracted from chemically transformed mouse fibroblasts and introduced, via the procedure of transfection, into untransformed mouse fibroblasts. The appearance of foci of transformed cells in the latter indicated the transmission of a transforming gene from the donor to the recipient cells, indicating that chemical carcinogens could indeed generate a mutant, cancer-causing gene.

carcinoma carried a point mutation—a single nucleotide substitution—that distinguished it from its counterpart proto-oncogene.[9-11] This genetic alteration, clearly a somatic mutation, sufficed to convert a normally benign proto-oncogene into a virulent oncogene.

Within months, yet other activated oncogenes were found in human tumors by using DNA probes prepared from a variety of retrovirus-associated oncogenes. The *myc* oncogene, initially associated with avian myelocytomatosis virus, was found to be present in increased gene copy number (i.e., amplified) in some human hematopoietic tumors[12]; in yet others, *myc* was activated through a chromosomal translocation that juxtaposed its coding sequences with those of immunoglobulin genes, thereby placing the expression of the *myc* gene under the control of these antibody genes rather than its own normal transcriptional control elements.[13] These discoveries extended and solidified a simple point: a common repertoire of proto-oncogenes could be activated either by retroviruses (usually in animal tumors) or by somatic mutations (in human tumors). The activating mutations involved either base substitution, amplification in gene copy number, or chromosomal translocation.

Multistep Tumorigenesis

The discoveries of mutant, tumor-associated oncogenes in human tumors led to a simple model of cancer formation. Mutagenic carcinogens entered into cells of a target tissue and mutated a proto-oncogene. The resulting oncogene then induced the now-mutant cell to initiate a program of malignant growth. Eventually, years later, the progeny of this mutant founder cell formed a large enough mass to become a macroscopically apparent tumor.

While satisfying conceptually, this simple model of cancer formation clearly conflicted with a century's worth of histopathologic analyses, which had indicated that tumor formation is really a multistep process, in which initially normal cell populations pass through a succession of intermediate stages on their way to becoming frankly malignant. Each of these intermediate stages contains cells that were more aberrant than those seen in the preceding steps. This body of observations persuaded many that the formation of a malignancy depended on a succession of phenotypic changes in the cells forming these various growths. Quite possibly, each of these shifts in cell phenotype reflected a change in the underlying genetic makeup of the evolving pre-malignant cell population. Such a multistep genetic model of tumor progression stood in direct conflict with the single-hit model of transformation that was suggested by the discovery of the point-mutated *ras* oncogene.

By 1983, one solution to this dilemma became apparent. In that year, experiments showed that a single introduced oncogene could not transform fully normal rat cells into ones that were tumorigenic. Two and maybe even more oncogenes seemed to be required to effect this conversion.[14,15] For example, whereas an introduced *ras* oncogene could not transform normal embryo cells into tumor cells, the co-introduction of a *ras* plus a *myc* oncogene, or a *ras* plus an adenovirus E1A oncogene, succeeded in doing so. It appeared that such pairs of oncogenes collaborated with one another to induce the full malignant transformation of normal cells (Figure 1-4, *A*). Moreover, this experiment suggested that human tumors carried two or more mutant oncogenes that collaborated with one another to orchestrate the many aberrant phenotypes associated with highly malignant cells.

Observations such as these pointed to a new way of conceptualizing the multistep tumorigenesis long studied by the pathologists. It seemed plausible that each of the

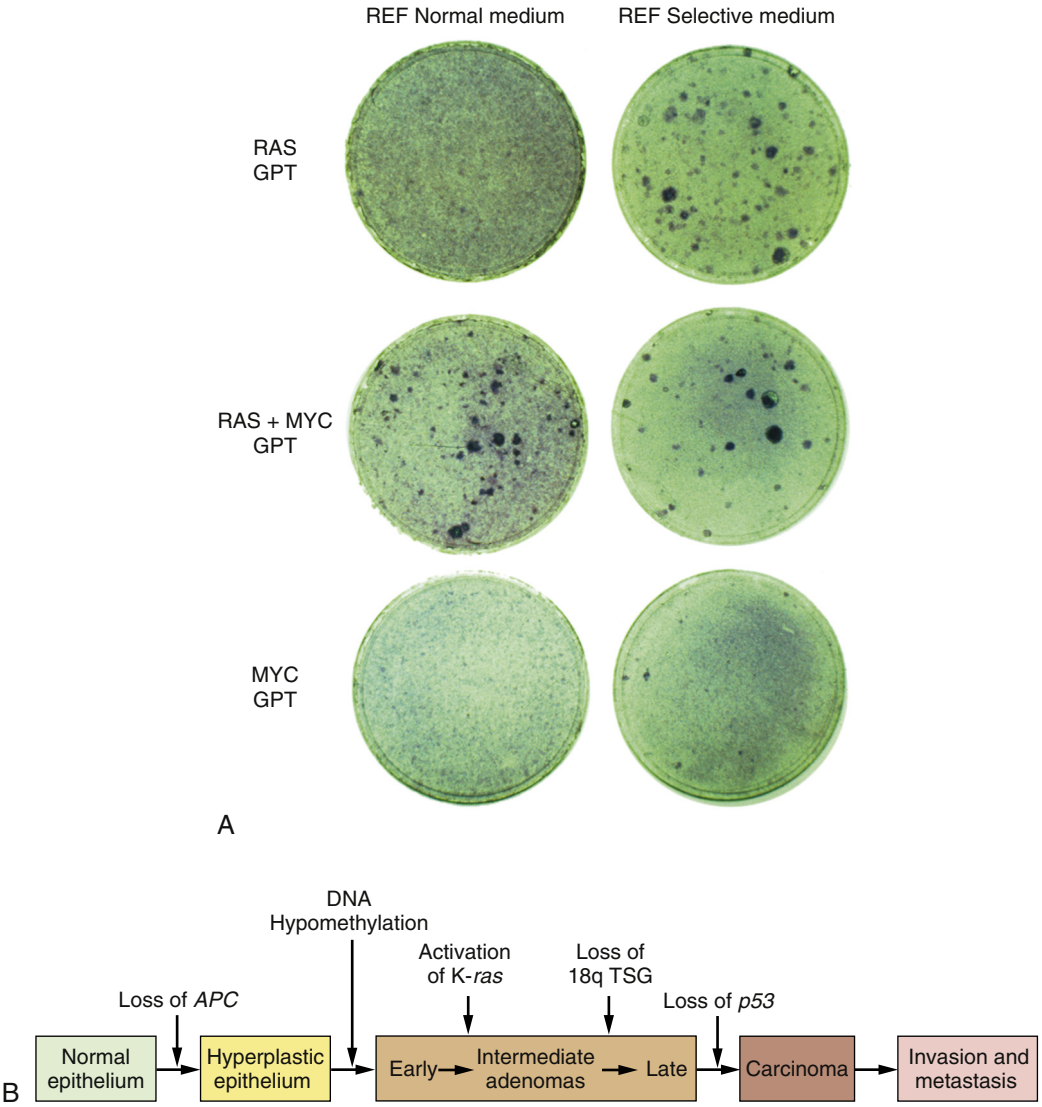


Figure 1-4 Multistep tumorigenesis in vitro and in vivo (A) The ability of oncogenes to collaborate to transform cells in vitro was illustrated in this 1983 experiment in which neither a *ras* nor a *myc* oncogene was found able to induce foci when introduced into early passage rat embryo fibroblasts (REFs). However, when the two were introduced concomitantly, transformation ensued, as indicated by the appearance of foci. This suggested that tumor progression in vivo might involve a succession of mutations that created multiple collaborating cellular oncogenes. **(B)** By 1989, analyses of the genomes of colonic epithelial cells at various stages of tumor progression revealed that the more progressed the cells were, the more mutations they had acquired. In fact, some of the indicated mutations involved inactivation of tumor suppressor genes, to be discussed later. *(A, from Land H, Parada LF, Weinberg RA.* Nature. *1983;304:596-602; B, courtesy B. Vogelstein.)*

histopathological transitions arising during tumor development occurred as a consequence of a new mutation sustained in the genome of an evolving, premalignant cell population (Figure 1-4, *B*). According to this thinking, tumor development was a form of Darwinian evolution, in which each successive mutation in a growth-controlling gene conferred increased proliferative potential and thus selective advantage on the cells bearing the mutant gene.[16,17] Ultimately, a multiply mutated cell bearing half a dozen or more mutant genes might exhibit all of the phenotypes associated with highly malignant cancer cells.

This mechanistic model was validated through the creation of transgenic mice. Cloned copies of mutant oncogenes, such as *ras* and *myc*, were introduced into the germlines of mice. These transgenes were structured so that the oncogene was placed under the control of a transcriptional promoter that ensured expression of the resulting "transgene" in a specific tissue or developmental stage. Now the presence of a mutant oncogene in a particular tissue could be guaranteed through the actions of an appropriately engineered transgene rather than being dependent on the random actions of mutagenic carcinogens.

In one highly instructive group of experiments, a *myc* or a *ras* oncogene was placed under the control of the mouse mammary tumor virus transcriptional promoter, which guaranteed its expression in the mammary epithelium of

the pregnant female mouse.[18] As anticipated, these mice contracted breast cancer at extremely high rates. This demonstrated that mutant oncogenes were far more than markers of cancer progression; indeed, they could actually play a causal role in driving tumor pathogenesis.

Significantly, the transgenic mice did not contract cancer rapidly in their mammary tissue even though a mutant oncogene was implanted and expressed in virtually all of the epithelial cells of their mammary glands. Instead, their mammary carcinomas arose with several months' delay, indicating that a second (and perhaps third) alteration was required in addition to the activated transgene before mammary epithelial cells launched a program of malignant growth. The nature of this additional alteration(s) was not always clear, but it almost certainly involved stochastic somatic mutations striking the mammary epithelial cells, creating mutant growth-controlling genes that collaborated with the transgene to trigger the outgrowth of malignant cell clones. In the years that followed, this work was extended to many types of human tumors, the cells of which were found to possess multiple mutant genes that contributed to tumor formation.

The Discovery of Tumor Suppressor Genes

The model of multistep tumorigenesis implied that a tumor cell carries two or more mutant oncogenes, each activated by somatic mutation during one of the stages of tumor development. However, experimental validation of this model initially proved to be difficult. Most attempts at detecting mutant oncogenes in human tumor genomes yielded a *ras* or perhaps a *myc* oncogene, but rarely were two mutant oncogenes found to coexist in the genomes of human tumor cells. This left two logical alternatives. Either the genome of a typical human tumor cell did not contain multiple mutated genes, as the multistep model of cancer suggested, or there were indeed multiple mutated cancer-causing genes in tumors, but many of these were not oncogenes of the type that had been studied intensively in the 1970s and early 1980s.

In fact, there were candidate genes waiting in the wings. These others operated in a fashion diametrically opposite to that of the oncogenes: they seemed to prevent cancer rather than favoring it and came to be called "tumor suppressor genes." Several independent lines of evidence led to the discovery and characterization of these genes.

Experiments using cell hybridization initiated by Henry Harris in Oxford provided the first indication of the existence of these suppressor genes.[19] These cell hybridizations involved the physical fusion of two distinct types of cells that were propagated in mixed cultures. The conjoined cells would form a common hybrid cytoplasm and ultimately pool their chromosomes, yielding a hybrid genome.

Often these cell hybridizations involved the fusion of cells with two distinct genotypes. In some of these experiments, tumor cells were fused with normal cells. The motive here was to see which genome would dominate in determining the behavior of the resulting hybrids. Counter to the expectations of many, the resulting hybrid cells turned out, more often than not, to be nontumorigenic.[19] This indicated that the genes present in the normal genome dominated over those carried in the cancer cell. In the language of genetics, the normal alleles were dominant, whereas the cancer cell–associated alleles were recessive. (More properly, the alleles present in the cancer cell created a phenotype that was recessive to the normal cell phenotype.)

This unanticipated behavior could most easily be rationalized by assuming that normal cells carried certain growth-normalizing genes, the presence of which was needed to maintain normal proliferation. Cancer cells seemed to have lost these genes, ostensibly through mutations that resulted in inactivated versions of the genes present in normal cells. When reintroduced into the cancer cells via cell fusion, the normal alleles reimposed control on the cancer cells, restoring their behavior to that of a normal cell. In effect, these growth-normalizing genes suppressed the tumorigenic phenotype of the cancer cells and were, for this reason, termed *tumor suppressor genes* (TSGs).

In their normal incarnations, the TSGs seemed to constrain growth, unlike the proto-oncogenes, which seemed to be involved in promoting normal proliferation. Inactivated, null alleles of TSGs were found in tumor cell genomes in contrast to the hyperactivated alleles of proto-oncogenes (i.e., oncogenes) found in these genomes.

The study of retinoblastoma, the childhood eye tumor, converged on these cell hybridization studies in a dramatic way. This work had been pioneered by Alfred Knudson, who, beginning in the early 1970s, studied the genetics of this rare tumor. Knudson learned much by comparing the two forms of this cancer: sporadic retinoblastoma, which seemed to be due exclusively to accidental somatic mutations, and familial retinoblastoma, which appeared, like many familial cancers, to be due to the transmission of a mutated gene in the germline.

Knudson's analysis of the kinetics of retinoblastoma onset persuaded him that a common set of gene(s) operated to generate both kinds of tumors.[20,21] Although the nature of these genes eluded him, their number was clear. Sporadic retinoblastomas seemed to arise following two successive somatic mutations affecting a lineage of cells in the retina. The triggering of familial retinoblastomas seemed to require only a single somatic mutation. Knudson speculated that in these familial tumors, a second mutated gene was required

to trigger tumorigenesis and that this gene was already present in mutant form in all the cells of the retina, having been inherited in mutated form from a parent of the affected child.

For the cancer geneticist, Knudson's most important concept was the notion that a retinal cell needed to lose two mutant genes before it was transformed into a tumor cell. Sometimes one of the two mutant null alleles was contributed by the germline; more often, both genes arose through somatic mutation. However, the nature of these genes and the mutations that recruited them into the tumorigenic process remained elusive. Finally, in 1979, karyotypic analysis of a retinoblastoma revealed an interstitial deletion in the q14 band of chromosome 13.[22] Later work revealed that this resulted in the loss of a gene, termed *RB*. Hence, one of the two mutational events needed to make a retinoblastoma involved the inactivation of an *RB* gene copy, in this particular case through the wholesale deletion of the chromosomal region carrying the *RB* gene.

By 1983, the nature of the second mutational event became clear: it involved the loss of the second, hitherto intact copy of the *RB* gene.[23] Hence, the two mutational events hypothesized by Knudson involved the successive inactivation of the two copies of this gene. Suddenly, the need for two mutations became clear: The first mutation left the cell with a single, still-intact copy of the *RB* gene, which was able, on its own, to continue programming normal proliferation. Only when this surviving gene copy was eliminated from the cell genome did runaway proliferation begin (Figure 1-5). Thus, mutations that inactivate an *RB* gene copy create alleles that function recessively at the cellular level. Only when both wild-type alleles are lost through various mutational mechanisms does a retinal cell begin to behave abnormally.

The *RB* gene became the paradigm for a large cohort of similarly acting TSGs that suffer inactivation during tumor progression. These TSGs are scattered throughout the cell genome and act through a variety of cell-physiologic mechanisms to control cell proliferation.[24] They are united only by the fact that they control proliferation in a negative way, so that their loss permits uncontrolled cell multiplication to proceed.

The discovery of the *RB* gene gave substance and specificity to the genes that Harris had postulated from his cell fusion experiments. Equally important, they opened the door to understanding a variety of familial cancer syndromes. In the case of *RB*, inheritance of a mutant, defective allele predisposes to retinoblastoma early in life with more than 90% probability. Inheritance of a defective allele of the *APC* TSG predisposes with high frequency to adenomatous polyposis coli syndrome and thus to colon cancer. The presence of a mutant *TP53* gene in the germline leads to increased rates of tumors in a number of organ sites, including sarcomas and

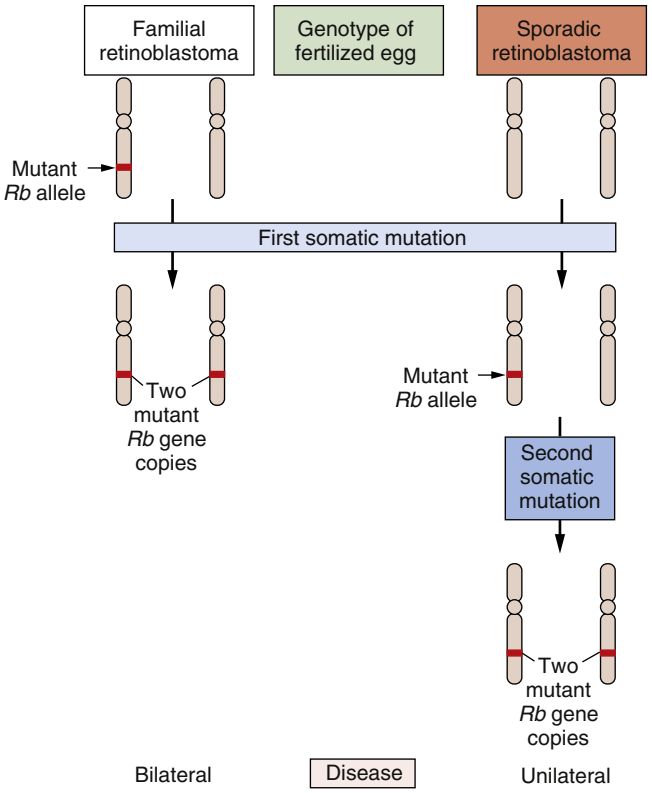


Figure 1-5 Genetics of retinoblastoma development The development of retinoblastomas requires the successive inactivation of two copies of the chromosomal *RB* gene. In the case of familial retinoblastomas, one of the two copies of this gene is already mutated in one or another gamete and is transmitted to the offspring, who is therefore heterozygous at this locus in all cells of the body; subsequent loss, through somatic alterations, of the surviving wild-type gene copy leaves a retinal cell with no functional copies of this gene, enabling tumor formation to begin. In sporadic retinoblastomas, the conceptus is genetically wild type; however, two successive somatic mutations occurring in a lineage of retinal precursor cells leaves some of these cells, once again, without functional *RB* gene copies, and as before permits retinoblastoma tumorigenesis to begin.

carcinomas, yielding the Li-Fraumeni syndrome. More than two dozen heritable cancer syndromes have been associated with germline inheritance of defective TSGs.[25,26]

In each case, the inheritance of a mutant, functionally defective TSG allele obviates one of two usually required somatic mutations. Because an inactivating somatic mutation represents a low-probability event per cell generation, the presence of an already-mutant inherited TSG allele enormously accelerates the overall kinetics of tumor formation. As a consequence, the likelihood of a tumor arising during the course of a normal lifespan is enormously increased.

The search for TSGs has been difficult, as their existence only becomes apparent when they are absent from a cellular genome. However, one peculiarity of TSG genetics has greatly aided the discovery of these genes. This involves the genetic mechanisms by which the second copy of a TSG is lost. In principle, two independent somatic mutations could successively inactivate the two copies of a TSG,

thereby liberating a cell from the growth-constraining influences of this gene. However, each of these mutations normally occurs with a low probability—perhaps 10^{-6} per cell generation. The likelihood of both mutations occurring is therefore roughly 10^{-12} per cell generation, an extremely low probability. (Actually, because cancer cell genomes become progressively destabilized as tumors develop, this probability is usually higher.)

In fact, evolving premalignant cell populations carrying a single, already-inactivated TSG copy often resort to another genetic mechanism to eliminate the second, still-intact copy of this TSG. They discard the chromosomal arm (or chromosomal region) carrying the still-intact TSG copy and replace it with a duplicated copy of the chromosomal region carrying the mutant, already-inactivated TSG copy. All this is achieved via the exchange of genetic material between paired homologous chromosomes.

The end result of these genetic gymnastics is the duplication of the mutant TSG copy. Thus, the TSG goes from a heterozygous state (involving one mutant and one wild-type gene allele) to a homozygous state (involving two mutant gene copies). Almost always, the chromosomal region flanking the TSG suffers the same fate. Consequently, known genes as well as other genetic markers within this flanking region that were initially present in a heterozygous configuration now become reduced to a homozygous configuration. This genetic behavior has motivated cancer geneticists to analyze the genomes of human tumor cells, looking for chromosomal regions that repeatedly suffer loss of heterozygosity (LOH) during tumor progression. Such LOHs represent presumptive evidence for the presence of TSGs in these regions whose second wild-type copies have been eliminated by LOH during the course of tumor development. Once such a region is localized to a chromosomal region, several currently available gene molecular strategies can be exploited to further narrow the chromosomal domain carrying the TSG and ultimately to isolate the TSG through molecular cloning.

The existence of many dozen still-unknown TSGs is suspected because of the documented LOH affecting specific chromosomal regions of various types of human tumor cells. The effort to identify and clone these genes is being greatly facilitated by efforts such as those included in the International Cancer Genome Consortium and the Cancer Genome Anatomy Project (TCGA). Nonetheless, the successful identification and cloning of a significant cohort of TSGs has already provided one solution to a major puzzle posed earlier. As mentioned, although human tumor cells were hypothesized to carry a number of distinct, mutated growth-controlling genes, most tumors appeared to carry only a single activated oncogene. We now realize that many of the other targets of mutation during tumor progression are TSGs. Their inactivation collaborates with the activated oncogenes to create malignant cells and thus tumors. In the widely cited study of human multistep tumor progression—that described in colonic tumors by Vogelstein and his coworkers—the mutation of a K-*ras* oncogene is accompanied by mutations of the *APC* and *TP53* TSGs and a third TSG that maps to chromosome 18.[27] This evidence, together with a wealth of genetic studies reported subsequently, indicates that TSGs are inactivated even more frequently than oncogenes are activated during the course of forming many types of human tumors. Importantly, the inactivation of TSGs often phenocopies the cell-biological effects of oncogenes. This means that the inactivation of TSGs is as important to the biology of tumor progression as oncogene activation.

Unexpectedly, the discovery of TSGs also made it possible to understand how a variety of DNA tumor viruses succeed in transforming the cells that they infect. Unlike retroviruses, these DNA viruses carry oncogenes that have resided in their genomes for millions, and likely hundreds of millions, of years. Any connections with antecedent cellular genes, to the extent they once existed, were obscured long ago by the extensive remodeling that these oncogenes underwent while being carried in the genomes of the various DNA tumor viruses. Independent of their ultimate origins, it was clear in the 1980s that the oncogenes (and encoded oncoproteins) were deployed by DNA viruses to perturb key components of the normal cellular growth-controlling circuitry. However, the precise control points targeted by these viral oncoproteins remained obscure.

In the late 1980s, it was learned that a number of DNA tumor virus oncoproteins bind to the products of two centrally important TSGs, pRB and p53.[28,29] For example, the large T oncoprotein of SV40 binds and sequesters both the p53 and pRB proteins of infected host cells; the E6 and E7 oncoproteins of human papillomaviruses target p53 and pRB, respectively. As a consequence, a virus-infected cell is deprived of the services of these two key negative regulators of its proliferation. Indeed, these virus-mediated inactivations closely mimic the state seen in many nonviral tumors that have been deprived of pRB and p53 function by somatic mutations striking the TSGs specifying these two proteins. So the transforming mechanisms used by these viruses could be rationalized by referring to the same genes and proteins that were known to be inactivated by mutational mechanisms in many types of spontaneous, nonviral human tumors. Importantly, these findings reinforced the notion that a single, central growth-regulating machinery operating in all types of cells suffers disruption by a variety of ostensibly unrelated genetic mechanisms, leading eventually to the formation of cancers.

The activation of oncogenes and the loss of TSGs together explain many of the phenotypes that one associates with cancer cells. These cells are able to grow without attachment to solid substrate, the aforementioned phenotype of anchorage

independence, and they are able to grow on top of one another, which is manifested in culture as the loss of contact inhibition. Moreover, when compared to normal cells, cancer cells exhibit a greatly reduced dependence on mitogens and an ability to resist the antiproliferative effects of growth-inhibitory signals, such as those conveyed by transforming growth factor-β (TGF-β). Alterations of oncogenes and tumor suppressor genes can be invoked to explain these neoplastic cell traits.

Arguably the most interesting trait of cancer cells is their ability to resist a variety of stimuli and stresses that would cause normal cells to activate the cell-suicide program termed *apoptosis*. The fact that virtually all tumor cells have developed various types of resistance to apoptosis indicates that severe pro-apoptotic stresses are experienced repeatedly as normal cells evolve progressively toward a malignant phenotype and that an ability to resist these stresses is strongly selected during this evolution. Thus, changes in the complex array of genes that control entrance in the apoptotic program are frequently demonstrable within tumor cells. Although these genes are specialized in regulating a discrete cancer cell phenotype (apoptosis), they behave operationally like oncogenes and TSGs, i.e., the activation of some of these confers a resistance to apoptosis as does the inactivation of yet others.

Guardians of the Genome

As mentioned previously, the somatic mutations that activate oncogenes or inactivate TSGs are relatively rare events in the life of a cell, occurring perhaps at a rate of 10^{-6} per cell generation. This low mutation frequency represents an important barrier to the development of neoplasia.[30] If cells require multiple mutations in order to progress to a fully malignant growth state, the probability of the entire constellation of mutations occurring within a cell lineage during a normal human lifespan is extremely low. This provides a partial explanation for the fact that we humans develop relatively few cancers during lifespans in which the cells in our bodies undergo more than 10^{16} divisions, each of which represents an opportunity for a genetic disaster.

As described earlier, the inheritance of a mutant growth-controlling gene obviates one of the normally required, rare somatic mutational steps. In doing so, it allows a population of premalignant cells to leapfrog over one of the barriers that usually block its progression toward malignancy. The consequence is the greatly increased risk of certain tumors that characterizes familial cancer syndromes.

However, there is at least one other route by which this multistep tumor pathogenesis can be accelerated: if the rate of gene mutation per cell generation is greatly increased, the time required for a population of cells to surmount all of the mutational hurdles and progress to full-blown malignancy will be correspondingly reduced. As a consequence, the probability of cancer striking during a normal lifespan will be greatly increased.

Xeroderma pigmentosum (XP) is the most thoroughly studied of the inborn cancer susceptibility syndromes that are attributable to greatly increased mutational frequency. Those suffering from XP show abnormally high sensitivity to ultraviolet (UV) radiation, which evokes squamous cell skin carcinomas and melanomas at exposed sites at a high rate. Like the rest of us, XP patients sustain large numbers of mutational events in their skin cells created by ultraviolet photons. In the skin cells of most humans, the pyrimidine dimers created by UV radiation are quickly excised from the damaged DNA and the initial, wild-type nucleotide sequence is restored, thereby erasing all traces of the mutation; this removal of DNA lesions is achieved by a cohort of DNA repair proteins that are specialized to effect this particular alteration of DNA structure. (In the event that skin cells exhibit widespread genomic damage that overwhelms the ability of its DNA repair apparatus to restore normal genome sequence, the cell may opt for another response, apoptosis, as discussed later.) In the XP patient, one or another essential component of this specialized DNA repair apparatus is absent or defective.[31] As a consequence, altered DNA sequences are transmitted to the progeny of the initially irradiated cell, resulting in large numbers of mutations in their genomes. Hence, the effective mutation rate (the number of initially induced mutations minus those that are repaired) increases enormously.

XP represented only the first of the familial cancer syndromes that has been attributable to defective DNA repair. In this particular syndrome, mutational damage is inflicted by an exogenous mutagen—UV radiation. We now know that a variety of other familial cancer syndromes are also attributable to defects in one or another component of the complex apparatus that maintains the integrity of our genome. In many of the more recently characterized cancer syndromes, the initial mutational damage is of endogenous origin, being inflicted by malfunctioning of normal cellular processes, including the mutations that result from mistakes in DNA replication and from the actions of endogenously generated mutagens, such as reactive oxygen species.

The ataxia telangiectasia syndrome, which includes, among its presentations, the development of certain tumors, is also due to defective DNA repair.[31,32] In hereditary nonpolyposis colon cancer (HNPCC), the apparatus that recognizes recently made mistakes in DNA replication, often termed the *mismatch repair apparatus*, is defective.[33,34] At least four different inherited subtypes of HNPCC have been described; each of these is due to defects in one or another component of the complex multicomponent system that recognizes and erases DNA copying mistakes as well as other

lesions that are occasionally inflicted on the cell genome. In the cells of HNPCC patients, one sees widespread genomic instability, the direct results of this defective DNA repair. The resulting genetic damage seems to affect all genes with equal frequency and thus the target proto-oncogenes and TSGs that participate in the formation of non-HNPCC colon cancers. As a consequence, the entire multistep process of colon cancer progression is greatly accelerated. Unexplained at present is why this genetic defect specifically afflicts the colon rather than causing elevated rates of cancer incidence in many sites throughout the body.

Many familial breast cancers have more recently been associated with inheritance of mutant versions of the *BRCA1* and *BRCA2* genes.[35] These were initially thought to be TSGs, but the peculiar behavior of the mutant alleles of these genes suggested otherwise. Mutant alleles of the *BRCA1* and *BRCA2* genes were found to be inherited in the germlines of affected individuals; however, sporadic mammary tumors rarely showed mutant alleles. Recent biochemical and cell biological experiments suggest that both these genes specify proteins that participate in the repair of double-strand DNA breaks. It remains unclear why the inheritance of defective alleles of either of these genes predisposes individuals specifically to breast and ovarian tumors.

There is increasing evidence that a breakdown of DNA repair capability accompanies the formation of the great majority of human tumors. These losses may occur through somatic mutation of DNA repair genes or, perhaps more frequently, through epigenetic mechanisms, such as DNA methylation (see later discussion), that succeed in repressing the expression of these repair genes, thereby depriving cells of the vital functions encoded by these genes.

Epigenetic Mechanisms Leading to Loss of Gene Function

As described earlier, the functions of two major classes of cellular genes are lost during the course of tumor progression—TSGs and DNA repair genes. It is highly likely that the development of the great majority of human tumors depends on these losses. Moreover, the portrayal of cancer as a genetic disorder, as developed previously, would suggest that these genes and their vital functions are lost through various mechanisms of somatic mutation. After all, mutations are by definition heritable, and thus the progeny of a cell that has initially acquired growth advantage through some somatic mutation will be similarly benefited, leading to the progressive expansion of clones of such mutant cells.

Following this logic, the phenotypic changes that occur during the course of tumor progression need to be heritable. In fact, there is a mechanism of heritability that does not depend on genetic alterations, i.e., on alterations of nucleotide sequence in a cell's genome. This mechanism depends on the methylation of the cytidine residues present in CpG dinucleotide sequences that are found in proximity to the promoters of various genes or by modification of histones in chromatin. Methylation or modification of histones often results in major shifts in the configuration of nearby chromatin and in the shutdown of the expression of nearby genes—the process of transcriptional repression.

When a DNA segment containing a methylated CpG is replicated, the complementary CpG in the newly synthesized daughter DNA strand is initially unmethylated. However, soon after this daughter strand is formed, "maintenance" DNA methylases recognize the hemimethylated DNA and attach a methyl group to the recently formed CpG residue, thereby ensuring that both CpGs are now methylated (Figure 1-6). This scheme ensures that DNA methylation events, and thus associated repression of certain genes, can be transmitted from parent to daughter cells with high fidelity. Hence, genes may be inactivated in a heritable fashion without any change in their nucleotide sequence.

In fact, the mechanisms that control DNA methylation result in the inactivation of genes at higher rates per cell generation than those involving somatic mutations. This leads to the obvious conclusion that the functions of TSGs and DNA repair genes are likely to be lost more frequently through DNA methylation than mutation, a notion that

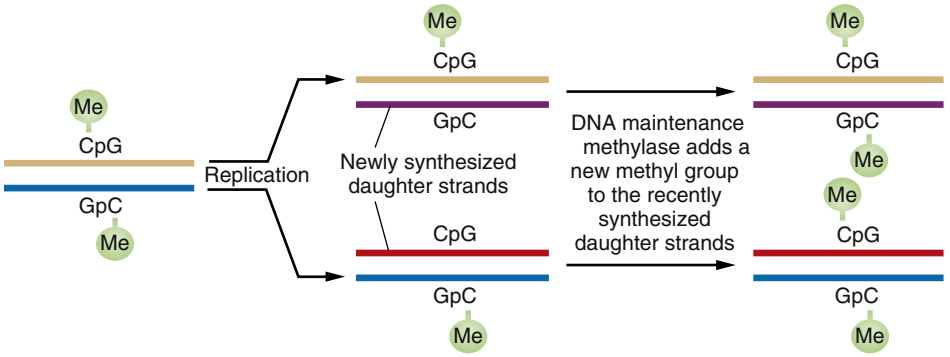


Figure 1-6 Perpetuation of CpG methylation following DNA replication When DNA methylated at CpG residues is replicated, the newly formed daughter strands initially lack methyl groups on the CpG sites complementary to those methylated sequences in the parental DNA strands. However, shortly after replication, maintenance methylases add methyl groups to the newly synthesized CpG sites, ensuring the transmission of the methylated state from one cell generation to the next. Such methylation is often associated with the repression of gene transcription.

is borne out by extensive studies of the genomes of human tumor cells.[36] Indeed, it now seems likely that individual tumor cell genomes bear many dozens if not hundreds of methylated genes. Most of these genes are likely be methylated as a consequence of the relaxed controls on DNA methylation that seem to operate within cancer cells; most such genes are bystanders, i.e., their loss is not functionally important for the cancer cell phenotype and their loss has not conferred selective advantage on the cells that carry them. However, a number of key TSGs and DNA repair genes have indeed been found to be methylated frequently in various types of human cancer cell genomes, and it is clear that the loss of gene function through promoter methylation is as effective in driving tumor progression as the somatic mutations that have been described extensively here.

Moreover, the modification of histones also alters chromatin and gene expression. Recent genome sequencing efforts have uncovered mutations in genes whose products play key roles in maintaining or modifying histone marks. For example, mutations in adenine-thymine (AT)-rich interactive domain-containing protein 1A (ARID1A),[36,37] a protein involved in chromatin remodeling, is mutated in more than 50% of ovarian clear cell cancers, and multiple chromatin-modifying enzymes including PBRM1 are mutated in a large fraction of renal clear cell cancers. These findings provide a link between the genetic and epigenetic origins of cancer. Hence, cancer pathogenesis is a disorder of genes and gene function, but it does not always depend on genetic alterations, because the epigenetic regulation of genes contributes as frequently, if not more frequently, to tumor formation.

Immortalized Proliferation

Yet another phenotype of cancer cells—their ability to grow and divide indefinitely—does indeed depend on changes in DNA structure and is, in this sense, a genetically determined trait. This unlimited proliferative ability, often termed *cell immortality*, stands in stark contrast to the limited proliferative ability of normal cell populations. Thus, when placed into culture, many types of cancer cells are able to proliferate indefinitely, in contrast to the behavior of normal cells, which cease proliferation after a limited, ostensibly predetermined number of doublings. This phenomenon of finite replicative potential suggests the workings of some type of generational clock that tallies the number of cell divisions through which cell lineages have passed since they resided in the early embryo and then informs cells in these lineages when their allotment of doublings has been exhausted. In response to this alarm, cell populations become "senescent," and if they overcome or circumvent senescence, will multiply further until they enter into a state of "crisis," in which almost all of them die.[38,39]

This limitation on replicative potential would seem to represent an important antineoplastic barrier erected by the organism. By limiting the number of successive replicative doublings its component cells may undertake, the organism erects a high barrier to the unlimited expansion of preneoplastic cell clones. Cancer cells must surmount this barrier in order to succeed in their agenda of unlimited growth and the formation of macroscopic tumors.

In fact, very different mechanisms govern the timing of the entrance of cell populations into senescence and into crisis. The senescence observed with cultured cells appears to be determined, in large part, by the conditions of their propagation in vitro. By necessity, the protocols developed for culturing cells create conditions that differ dramatically from those operating within living tissues. These discrepancies derive from the contents of the culture medium as well as the oxygen tensions experienced by cells within tissue culture incubators. As a consequence, cells suffer substantial physiologic stress when placed into culture, and cumulative cell-physiologic stress seems to be a major, if not the major, determinant of the triggering of senescence.

The mechanisms governing entrance into crisis are very different and do indeed involve, quite directly, the cell genome, more specifically the telomeres at the ends of all chromosomes. Evidence accumulated in recent years points to the telomeres as the molecular devices that tally cell generations and govern entrance into crisis. The ends of the telomeric DNA are not copied completely during each cycle of DNA replication because of an intrinsic limitation in the DNA polymerases responsible for the bulk of DNA replication. In addition, the ends of telomeric DNA are susceptible to the actions of exonucleases, which contribute to further erosion of telomeric DNA length. As a consequence, the telomeres shorten progressively as cell lineages pass through repeated cycles of growth and division (Figure 1-7). In normal cell lineages, this shortening eventually results in critically truncated telomeres. Without the protective effects of the telomeres, chromosomes undergo end-to-end fusion with resulting karyotypic instability and cell death. Hence, the progressive shortening of telomeres represents an effective molecular device for counting cumulative generational doublings.[38,39]

Cancer cell populations must overcome this limitation on their proliferation in order to proliferate extensively and generate macroscopic tumors. They do so by activating expression of the telomerase enzyme, which is able to restore and maintain telomeric DNA length, thereby reversing the effects of telomere erosion. Telomerase activity is detectable in almost all human tumors (approximately 90%) but is present at low or undetectable levels in the corresponding

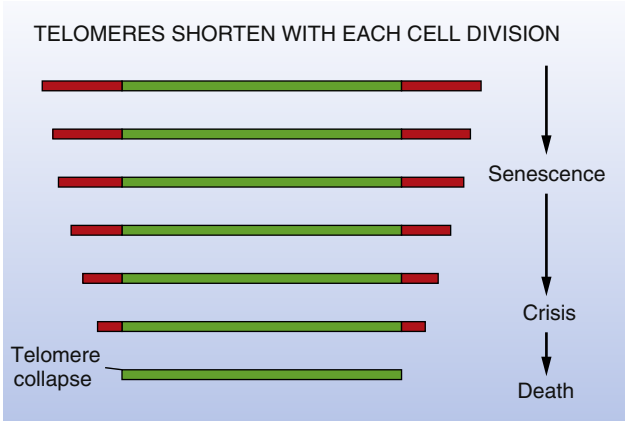


Figure 1-7 Telomere erosion and entrance into crisis In the absence of active intervention by the telomerase enzyme, the telomeres of human chromosomes shorten progressively during each round of cell growth-and-division, eventually losing so much length that they can no longer subserve their normal function of protecting the ends of chromosomal DNA from end-to-end fusions with other chromosomes. This leads to massive cell death, termed crisis, and occasionally, the emergence of a rare variant that has indeed acquired telomerase expression and is accordingly now able to repair and maintain telomeric DNA and thus telomeres. (Although the onset of senescence is indicated here as also being triggered by telomere shortening, it appears that it is largely due to cumulative cell-physiologic stresses sustained by cells both in vitro and in vivo.)

normal tissues. Accordingly, the genes that allow telomerase activation during tumor progression represent yet additional important genetic elements that are affected during the development of almost all human tumors. Importantly, however, the human telomerase gene, termed *hTERT*, is not itself the target of mutation. Instead, its expression is induced by a complex array of *trans*-acting transcriptional regulators, the MYC oncoprotein being one of these.

The critical contribution of telomerase to tumorigenesis is illustrated most dramatically by the protocols that enable the experimental transformation of normal human cells into tumor cells. By adding the *hTERT* gene to a cocktail of other introduced oncogenes, a variety of normal human cells can be converted to a tumorigenic state, as judged by their behavior following implantation into appropriate host mice.[40] The *hTERT* gene clearly affords such cells the ability to proliferate indefinitely; without its actions, cells fail to proliferate extensively in vitro and to form tumors in vivo.

Non-genetic Mechanisms Accelerating Multistep Tumor Progression

The descriptions of tumorigenesis, as developed here, lead to the notions that the functioning of normal cell genomes is progressively degraded by mutagenic mechanisms, promoter methylation, and telomerase erosion and that these mechanisms conspire to drive forward multistep tumor progression. An obvious corollary is that exposure to high levels of mutagenic agents is likely to serve as a major agent that stimulates human tumor formation. Indeed, since the initial experiments of Bruce Ames, such logic has inspired the search for the mutagens that are responsible for instigating human cancers.

In truth, with some notable exceptions, the search for the mutagenic carcinogens that drive human cancer pathogenesis has failed.[41] Tobacco smoke, with its high levels of mutagens, is clearly responsible for almost one-third of human cancers. In addition, the heterocyclic amine mutagens created by the cooking of red meat at high temperatures are attractive candidates for the agents causing many colon and possibly prostate cancers.

In general, however, the carcinogens responsible for most human cancer incidence have eluded identification, apparently because they do not function as mutagens. Instead, it has become increasingly apparent over the past two decades that the major determinants of human cancer incidence are various agents and conditions that operate as "tumor promoters." Thus, as illustrated by the classic experiments involving mouse skin cancers, tumor "initiators" are responsible for triggering the first step of multistep tumorigenesis by mutating certain target genes (e.g., H-*ras*), whereas promoters are responsible for driving the clonal expansion of already-initiated tumor cells, doing so through mechanisms that do not involve genetic damage. It seems increasingly likely that most of the determinants of human cancer incidence operate as promoters.

Possibly the most important promoting mechanisms involve chronic inflammation of tissues and the associated release of growth-stimulating factors by the irritated tissue. Moreover, many of the dietary determinants of tumor incidence would seem to function as promoters rather than as mutagenic initiators. If these notions are sustained by future research, this will mean that a complete understanding of cancer pathogenesis at the molecular level will require detailed elucidation of these non-genetic, tumor-promoting mechanisms.

Invasive and Metastatic Behaviors

In many individuals, the endpoint of multistep tumor progression involves, unfortunately, the acquisition by cancer cells of the ability to invade and to metastasize from the primary tumor to distant sites in the body—the manifestations of high-grade malignancy. Indeed, the metastases that are spawned by malignant tumors are responsible for 90% of cancer-associated mortality.

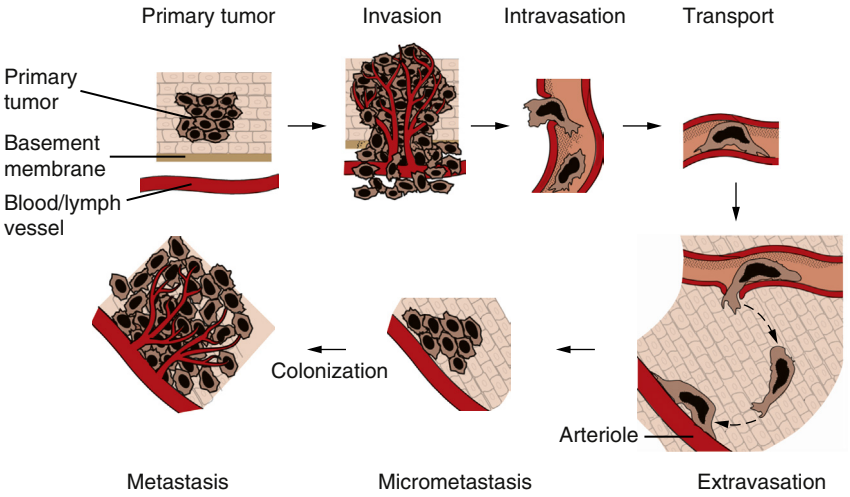


Figure 1-8 The invasion-metastasis cascade The invasion-metastasis cascade is a complex, multistep process through which cancer cells must pass in order to launch macroscopic tumor colonies at distant sites. These steps are executed relatively inefficiently, resulting in vast numbers of cells being disseminated from primary tumors with only a small number of cells being able to eventually form macroscopic metastases.

The formation of metastases is the result of a complex, multistep process that is often termed the *invasion-metastasis cascade* (Figure 1-8). Thus, cancer cells in the primary tumor acquire the ability to invade adjacent tissue, to enter into the vessels of the blood and lymphatic systems (intravasation), to travel in these channels to distant sites in the body, to escape from these vessels (extravasation) into nearby tissues, and to found small tumor colonies (micrometastases) in these tissues. On occasion, the cells forming a micrometastasis will acquire the ability to proliferate vigorously, resulting in the formation of a macroscopic metastasis—the process termed *colonization*.

The complexity of the invasion-metastasis cascade rivals that of the multistep process that leads initially to the formation of a primary tumor. This suggests, in turn, that cancer cells within a primary tumor must suffer a significant number of genetic alterations in order to acquire the ability to complete this cascade. Another alternative has presented itself, however, as the result of recent research on the malignant behavior of carcinoma cells. This alternative mechanism involves the actions of genes that are normally involved in programming certain key steps of early embryonic morphogenesis. In such steps of embryogenesis, epithelial cells, which are normally immobilized in various layers, undergo a profound change in their differentiation program and acquire many of the phenotypes of mesenchymal cells, including motility and invasiveness. This transdifferentiation program is termed the *epithelial-mesenchymal transition* (EMT).

As many as half a dozen transcription factors acting during various stages of early embryogenesis are capable of programming EMTs. These transcription factors have names such as Snail, Slug, Twist, Goosecoid, and SIP-1. Each of these is able to act pleiotropically to program an EMT and thereby is able to cause the repression of epithelial genes and the induction, in their stead, of mesenchymal genes. Increasing experimental evidence indicates that carcinoma cells exploit these early embryonic genes in order to execute most of the steps of the invasion-metastasis cascade.[42,43]

Expression of these embryonic genes seems to be induced by contextual signals that these carcinoma cells experience in the tumor microenvironment and that originate in the tumor-associated stroma. For example, TGF-β impinging on certain cancer cells is able to elicit the expression of several of the transcription factors that are capable, in turn, of programming an EMT. This suggests that the EMT program, and the enabling of the invasion-metastasis cascade, occurs because of a collaboration between the genotype of cancer cells and the contextual signals that these cells receive from the nearby microenvironment, more specifically from the activated stroma that is present in many primary tumors. Moreover, it suggests that certain carcinoma cell genotypes render these cells responsive to such stromal, EMT-inducing signals, whereas other genotypes leave the cancer cells unresponsive, indeed refractory, to these signals; our understanding of these genotypes is still fragmentary.

The discovery of these embryonic transcription programs and their resurrection by carcinoma cells greatly simplifies our conceptualization of the late stages of malignant progression. Rather than needing to acquire a number of distinct mutations in order to execute the various steps of the invasion-metastasis cascade, the genotypes of certain primary cancer cells allow them, in response to stromal signals, to activate long-dormant cell biological programs—EMTs. Once activated, this program seems to enable a carcinoma cell to complete most of the steps of the invasion-metastasis cascade. However, the last step—colonization—appears to involve an adaptation to the novel tissue microenvironment in which disseminated carcinoma cells have landed; such adaptation would not seem to be found among the multiple powers of the EMT program and would seem to acquire yet other changes that remain poorly understood.

Interestingly, the carcinoma cells forming a metastasis often recapitulate the histopathological appearance of the primary tumor, including its distinctive epithelial cell sheets and ducts. This would seem to be at variance with the notion that in order to metastasize, carcinoma cells must shed their epithelial characteristics and acquire, instead, mesenchymal ones. It seems plausible, however, that once carcinoma cells have disseminated and landed in distant tissue sites, they no longer encounter the mix of signals that were released by the activated stroma of the primary tumor and that led initially to their passing through an EMT. This new tissue microenvironment may therefore allow these cells to revert, via a mesenchymal-epithelial transition (MET) to the epithelial phenotype of their progenitors in the primary tumor, thereby generating once again epithelial histomorphology. Importantly, although passage through a partial or complete EMT may explain the malignant behavior of many carcinoma cells, it is less clear how tumors of other tissue origins, namely those arising in neuroectodermal, mesenchymal, and hematopoietic tissues, acquire these aggressive traits. The mechanisms enabling invasive and metastatic behaviors in these other neoplastic cell types remain elusive.

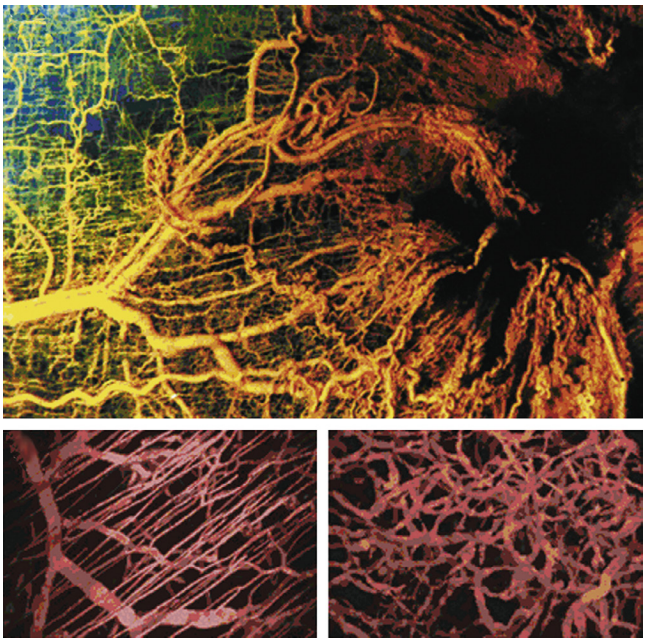


Figure 1-9 Tumor angiogenesis As tumors grow, they develop large networks of blood vessels through the process of angiogenesis. Seen here is a tumor *(black mass, right)* that has attracted blood vessels growing into it from adjacent normal tissue *(left)*. As is the case with most tumor-associated neovasculature, the new vessels developed here are tortuous and often end in dead ends *(right)*, in contrast to the normal vasculature seen here. *(Reproduced from Weinberg RA.* The Biology of Cancer. *New York, NY: Garland Science; 2007:562.)*

Other Phenotypes of Neoplasia

Many of the phenotypes of cancer cells are not readily explained by alterations in their proto-oncogenes and TSGs. Cancer cells acquire other aberrations that favor their growth in the complex environments of living tissues. Included among these is their ability to recruit blood vessels into tumor masses—the process of angiogenesis[44]—and, quite possibly, their ability to evade and overwhelm immune defenses.[45]

The process of tumor angiogenesis, like the EMT, involves a complex array of heterotypic interactions between cancer cells and their mesenchymal microenvironment (Figure 1-9). Indeed, this neoangiogenesis has become a subject of intensive investigation over the past decade, in part because the demonstrated dependence of tumors on vascularization represents an attractive target for therapeutic intervention through the creation and implementation of various antiangiogenic therapies. Thus, without adequate vascularization, cancer cells are limited to forming tumors of less than 1-mm diameter.

The processes of neovascularization depend on the heterotypic interactions of cancer cells with circulating endothelial precursor cells and with existing endothelial cells in the nearby stroma. Moreover, other regulators of this process include macrophages, myofibroblasts, and neutrophils, which may collaborate with the cancer cells to release angiogenic signals and thereby recruit endothelial cells and induce them to construct microvasculature. In addition, pericytes, which form the outer wall of most microvessels, must be recruited in order to ensure the assembly of well-constructed microvessels.

The role of the immune system in defending against the formation of various human tumor types remains a matter of great contention. Actually, in the case of virus-induced cancers, the protective role of the immune system is no longer debated, because of the abundant evidence that immunocompromised individuals suffer dramatically increased rates of virus-induced malignancies, including Kaposi's sarcoma, human papillomavirus–induced squamous cell carcinomas, and certain types of Epstein-Barr virus–induced hematopoietic disorders. In all of these cases, these functions can be readily rationalized by invoking the known antiviral effects of the immune system.

More challenging, however, are the actions of the immune system in reducing the incidence of tumors of nonviral etiology, which constitute more than 80% of the total tumor burden in the population. In these cases, it has been unclear how the immune system can recognize tumor cells as being of foreign origin and proceed to attack and eliminate them. That such attack often occurs is clear, however, as evidenced by the severalfold increased incidence of a variety of common tumors in patients who are immunocompromised

for various reasons, largely involving the preservation of organ transplants. This phenomenon provides hope that the immune system is indeed capable of recognizing and attacking nonviral tumors and that its powers can be exploited to serve as antitumor therapeutic modalities.

The molecular genetic paradigm described here has allowed us to understand the workings of the cancer cell in enormous detail. Thirty years ago, no one could have anticipated this explosion of knowledge. Genes have led to encoded proteins, and the study of these proteins has allowed us to elucidate complex regulatory circuits transmitting signals that flux through the cancer cell and control its proliferation, differentiation, and death.

The discovery of oncogenes has begun to have an impact in the clinic. Small-molecule inhibitors or antibodies directed against activated oncogenes, such as BRAF, BCR-ABL, and HER2, are now approved for the treatment of melanoma, chronic myelogenous leukemia, and breast cancer (Figure 1-10). In addition, large-scale efforts to characterize cancer genomes have confirmed the key role for many of the known oncogenes and tumor suppressor genes and uncovered new classes of potential cancer targets.[46-52] These efforts have also stimulated the development of strategies to perform genome characterization of patient samples. The extension of such efforts is likely to fundamentally alter the diagnosis and classification of cancers. Although it is clear that these initial molecularly targeted therapies will not lead to durable cures in most cases, with the greatly increased understanding of the genetic mechanisms of cancer pathogenesis, many novel ways of detecting and curing tumors are now, finally, within reach.

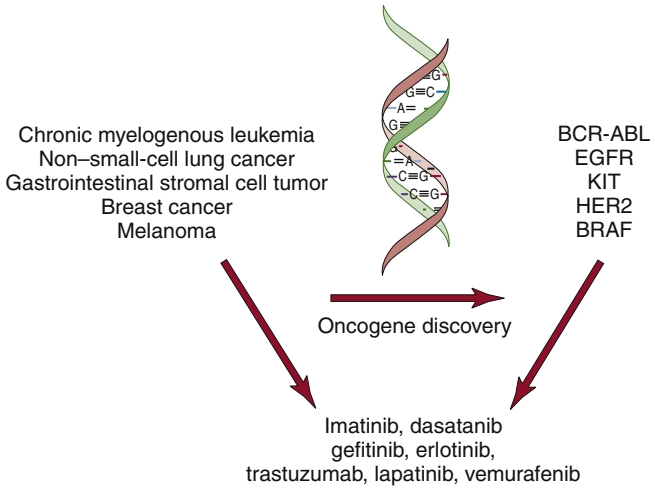


Figure 1-10 Molecularly targeted therapies The discovery of oncogenes in specific types of human cancers has led to the development of molecularly targeted inhibitors. These inhibitors show specificity for cancers that harbor the specific mutations.

Key References

1. Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, PA: Lippincott Williams & Wilkins; 2007.
3. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*. 2003;3(3):155-168.
36. Jones S, Wang TL, Shih IM, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330(6001):228-231.
37. Wiegand KC, Shah SP, Al-Agha OM, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med*. 2010;363(16):1532-1543.
46. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321(5897):1807-1812.
47. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318(5853):1108-1113.
48. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474(7353):609-615.
49. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455(7216):1061-1068.
50. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer*. 2004;91(2):355-358.
51. Campbell PJ, Stephens PJ, Pleasance ED, et al. Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. *Nat Genet*. 2008;40(6):722-729.
52. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. *Nature*. 2007;446(7132):153-158.

This page intentionally left blank

References

1. Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, PA: Lippincott Williams & Wilkins; 2007.
2. McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. *Proc Natl Acad Sci U S A*. 1975;72(12):5135-5139.
3. zur Hausen H. Viruses in human cancers. *Science*. 1991;254(5035):1167-1173.
4. Stehelin D, Varmus HE, Bishop JM, Vogt PK. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature*. 1976;260(5547):170-173.
5. Bishop JM. Cellular oncogenes and retroviruses. *Annu Rev Biochem*. 1983;52:301-354.
6. Weiss R, Teich N, Varmus H. *Molecular Biology of Tumor Viruses: RNA Tumor Viruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1985.
7. Shih C, Shilo BZ, Goldfarb MP, Dannenberg A, Weinberg RA. Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin. *Proc Natl Acad Sci U S A*. 1979;76(11):5714-5718.
8. Der CJ, Krontiris TG, Cooper GM. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. *Proc Natl Acad Sci U S A*. 1982;79(11):3637-3640.
9. Reddy EP, Reynolds RK, Santos E, Barbacid M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature*. 1982;300(5888):149-152.
10. Tabin CJ, Bradley SM, Bargmann CI, et al. Mechanism of activation of a human oncogene. *Nature*. 1982;300(5888):143-149.
11. Taparowsky E, Shimizu K, Goldfarb M, Wigler M. Structure and activation of the human N-ras gene. *Cell*. 1983;34(2):581-586.
12. Alitalo K, Schwab M. Oncogene amplification in tumor cells. *Adv Cancer Res*. 1986;47:235-281.
13. Leder P, Battey J, Lenoir G, et al. Translocations among antibody genes in human cancer. *Science*. 1983;222(4625):765-771.
14. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*. 1983;304(5927):596-602.
15. Ruley HE. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature*. 1983;304(5927):602-606.
16. Hunter T. Cooperation between oncogenes. *Cell*. 1991;64(2):249-270.
17. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194(4260):23-28.
18. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell*. 1987;49(4):465-475.
19. Harris H. Cell fusion and the analysis of malignancy. *Proc R Soc Lond B Biol Sci*. 1971;179(54):1-20.
20. Knudson Jr AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*. 1971;68(4):820-823.
21. Knudson AG. Two genetic hits (more or less) to cancer. *Nat Rev Cancer*. 2001;1(2):157-162.
22. Yunis JJ, Ramsay N. Retinoblastoma and subband deletion of chromosome 13. *Am J Dis Child*. 1978;132(2):161-163.
23. Cavenee WK, Dryja TP, Phillips RA, et al. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*. 1983;305(5937):779-784.
24. Weinberg RA. Tumor suppressor genes. *Science*. 1991;254(5035):1138-1146.
25. Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science*. 1997;278(5340):1043-1050.
26. Vogelstein B, Kinzler KW. *The Genetic Basis of Human Cancer*. New York, NY: McGraw-Hill; 1998.
27. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61(5):759-767.
28. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell*. 1997;88(3):323-331.
29. Nevins JR. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science*. 1992;258(5081):424-429.
30. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res*. 1991;51(12):3075-3079.
31. Friedberg EC, Walker GC, Siede W. *DNA Repair and Mutagenesis*. Washington, DC: ASM Press; 1995.
32. Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer*. 2003;3(3):155-168.
33. Heinen CD, Schmutte C, Fishel R. DNA repair and tumorigenesis: lessons from hereditary cancer syndromes. *Cancer Biol Ther*. 2002;1(5):477-485.
34. Modrich P, Lahue R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem*. 1996;65:101-133.
35. Venkitaraman AR. Cancer susceptibility and the functions of *BRCA1* and *BRCA2*. *Cell*. 2002;108(2):171-182.
36. Jones S, Wang TL, Shih IM, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330(6001):228-231.
37. Wiegand KC, Shah SP, Al-Agha OM, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med*. 2010;363(16):1532-1543.
38. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol*. 2000;1(1):72-76.
39. Shay JW, Zou Y, Hiyama E, Wright WE. Telomerase and cancer. *Hum Mol Genet*. 2001;10(7):677-685.
40. Hahn WC, Weinberg RA. Rules for making human tumor cells. *N Engl J Med*. 2002;347(20):1593-1603.
41. Gold LS, Ames BN, Slone TH. Misconceptions about the causes of cancer. In: Paustenbach DJ, ed. *Human and Environmental Risk Assessment: Theory and Practice*. New York, NY: John Wiley & Sons; 2002.
42. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays*. 2001;23(10):912-923.
43. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2(6):442-454.
44. Ferrara N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer*. 2002;2(10):795-803.
45. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3(11):991-998.
46. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321(5897):1807-1812.
47. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318(5853):1108-1113.
48. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474(7353):609-615.
49. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455(7216):1061-1068.
50. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer*. 2004;91(2):355-358.
51. Campbell PJ, Stephens PJ, Pleasance ED, et al. Identification of somatically acquired rearrangements in cancer using genome-wide massively parallel paired-end sequencing. *Nat Genet*. 2008;40(6):722-729.
52. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. *Nature*. 2007;446(7132):153-158.

Luca Grumolato and Stuart A. Aaronson

2

Oncogenes and Signal Transduction

Signaling: An Overview

Intercellular communication is critical to embryonic development, tissue differentiation, and systemic responses to wounds and infections. These complex signaling networks are in large part initiated by growth factors. Such factors can influence cell proliferation in positive or negative ways, as well as inducing a series of differentiated responses in appropriate target cells including survival, apoptosis, and differentiation. The interaction of a growth factor with its receptor by specific binding in turn activates a cascade of intracellular biochemical events ultimately responsible for the biological responses observed. Several classes of receptors are involved in transducing these extracellular signals. These include receptor tyrosine kinases, G-protein coupled receptors, and cytokine receptors. Cytoplasmic molecules that mediate these responses have been termed *second messengers*. The transmission of these biochemical signals to the nucleus leads to the altered expression of a wide variety of genes involved in mitogenic, survival, and differentiation responses.

As knowledge has accumulated in the area of signal transduction and the complexities increase, it is becoming apparent that overlap exists in cell signaling. This functional redundancy may be seen at several levels. The simplest example would be the fact that several different extracellular signals can lead to the activation of the same pathway. Physiologically, this may serve to allow a cell to respond to a variety of different situations or stresses while conserving some of the downstream machinery. Although some of the components of certain pathways may be common for two different stimuli, the ultimate physiological response may differ greatly because of the activation of a different repertoire of nuclear response elements. In addition, although redundancy may exist in terms of the ability of a stimulus to perturb a specific pathway, it is conceivable that the kinetics and magnitude of activation may differ, leading to distinct outcomes.

There is often redundancy among different isoforms of certain proteins or with members of particular gene families. This is illustrated by the fact that targeted disruptions of some genes fail to produce detectable phenotypes in mice, indicating that other proteins can compensate for their loss. An attractive explanation for this redundancy is that it serves as a fail-safe mechanism to ensure proper functioning in the face of damaging mutations that lead to a loss of function. Indeed, as discussed in more detail later, proteins within a family often have overlapping functions and may in some situations complement one another.

In order to effectively coordinate signaling cascades, nature has created a variety of molecules known as adaptor and scaffolding proteins.[1] These proteins play an integral role in intracellular signaling by recruiting various proteins to specific locations, as well as by assembling networks of proteins particular to a cascade. Adaptor proteins, through protein-protein interactions via specific motifs, provide a link between molecules of a signaling cascade and proteins such as receptor tyrosine kinases (RTKs; Figure 2-1). Adaptors can be docking proteins, which provide multiple binding sites on which effector molecules can attach, thereby expanding the magnitude of responses from an activated RTK. Scaffolding proteins also exist in signaling cascades and allow the formation of multienzyme complexes that are involved in a particular cascade. These are important for two reasons. The first is that the activation of a signaling cascade by a growth factor is an extremely rapid process and is not likely to occur as a result of proteins randomly floating in the intracellular milieu until they happen to come in contact with each other. Scaffolding proteins ensure the close proximity of the necessary components. The second reason is that several enzymatic components of a particular signaling cascade may be shared, although the substrates of each may differ. Thus, scaffolding proteins ensure the proper routing of signals by preventing unwanted cross talk between pathways.

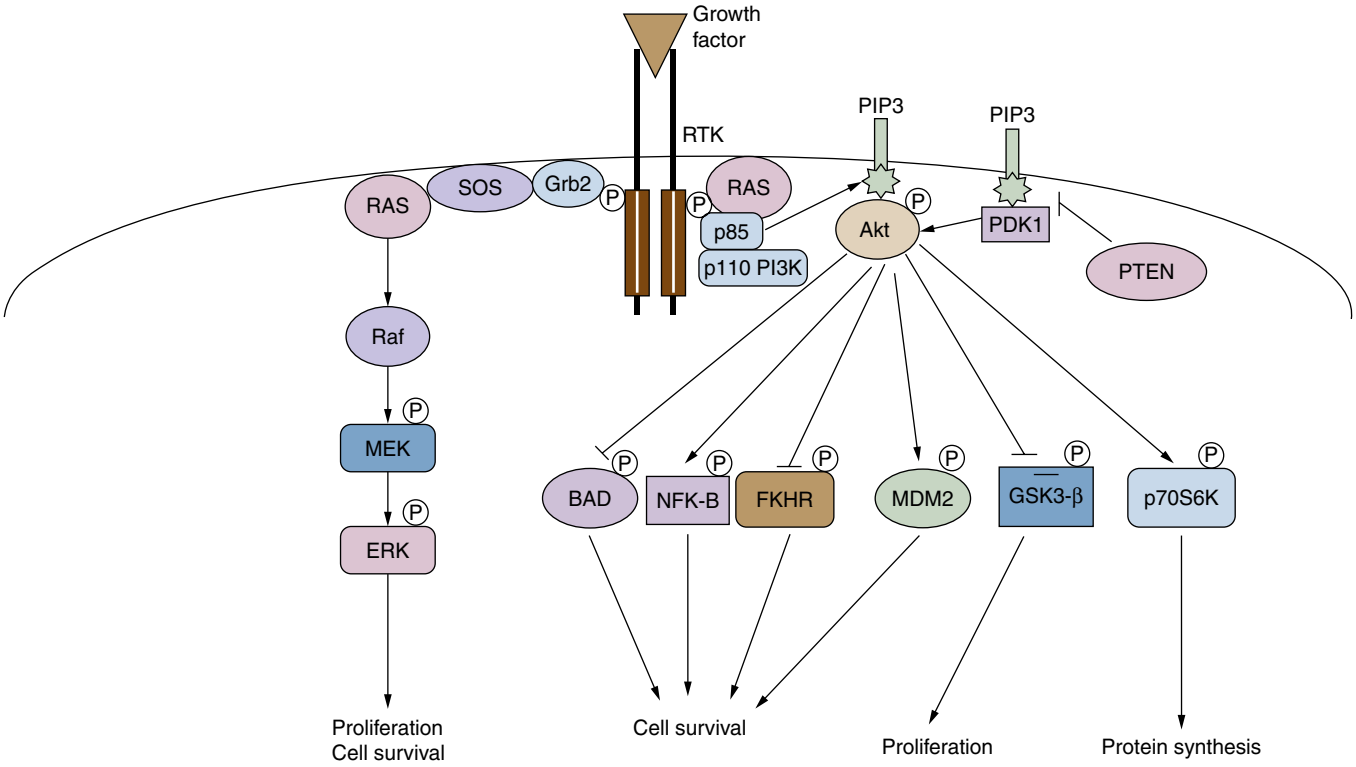


Figure 2-1 Receptor tyrosine kinase signaling in cancer Scheme for growth factor signaling through receptor tyrosine kinases.

Oncogenes

Oncogenes encode proteins that possess the ability to cause cellular transformation. These genes act in a dominant fashion, through either overexpression or activating mutations. There are several criteria that define cellular transformation. These include morphological changes, loss of contact inhibition, anchorage-independent growth, and the ability to form tumors when transplanted into nude mice. For example, under normal physiological situations, a growth factor binding to a receptor produces a very transient activation of a certain signaling cascade allowing tightly regulated responses such as proliferation to occur. When downstream components of these cascades are mutated in a way that causes them to be constitutively active, the signal is no longer transient and regulated but is aberrantly turned on in a continuous fashion. In addition to activating mutations, these genes can be activated by overexpression at levels much higher than in normal cells. Proto-oncogenes are commonly involved in cellular signaling, and specific examples are discussed later in the context of their roles in signal transduction.

Initially, it was believed that cellular transformation was caused solely by unregulated cell proliferation induced by activation of oncogenes. It is now known that although deregulated proliferation is most likely a necessary component for transformation, it is probably not sufficient, and other changes, such as modulation of cell survival functions, are critical as well. In fact, as discussed later, certain oncogenes function to modulate cell survival.

In the early 1980s, approaches aimed at identifying the functions of retroviral oncogenes converged with efforts to investigate normal mitogenic signaling by growth factors. Analysis of the predicted sequences of a number of retroviral oncogene products uncovered several with similarities to the prototype v-src product, whose enzymatic function as a protein kinase had been identified. Unlike many protein kinases, which phosphorylated serine and/or threonine residues, the v-src product was a protein kinase capable of specifically phosphorylating tyrosine residues.[2] Later efforts to identify oncogenes led to the discovery of the small GTPase Ras, which was unmasked as a transforming gene by transfection of tumor cell genomic DNA.[3]

Independent efforts to purify and sequence growth factors led to the discovery that the sequence of the platelet-derived growth factor (PDGF) B chain matched the predicted product of the transforming gene of simian sarcoma virus, designated v-sis.[4,5] The v-erbB gene of avian erythroblastosis virus, which predicted a v-src–related protein tyrosine kinase, was then found to represent a truncated form of the epidermal growth factor receptor (EGFR).[6] Independent evidence demonstrated that EGF triggering of its receptor resulted in tyrosine autophosphorylation.[7] Thus, a direct link between growth factors, receptors with tyrosine

kinase activity, and oncogenes was firmly established. The proliferation, differentiation, functional activity, and survival of cells can be affected by a wide array of other cytokines that signal through transmembrane receptors that lack protein tyrosine kinase activity. Because these signaling systems have also been implicated in malignant transformation, they are described in this chapter as well.

Signal Transduction by Protein Tyrosine Kinase Receptors

Membrane-spanning RTKs contain several discrete domains, including their extracellular ligand binding, transmembrane, juxtamembrane, protein tyrosine kinase, and carboxy-terminal tail domains.[8,9] Interaction of a growth factor with its receptor at the cell surface leads to a tight association, so that growth factors are capable of mediating their activities at very low concentrations. In the general model of RTK activation, ligand binding induces receptor dimer or oligomer formation associated with activation of the tyrosine kinase domain. However, the dimerization mechanism can be very different among the 20 families of RTKs, and the contribution of the ligands varies. For example, nerve growth factor (NGF) dimers directly mediate the coupling of two TrkA receptors, whereas EGF molecules do not contribute to the EGFR dimerization interface, but instead induce a conformational switch, which unmasks a dimerization domain in the EGFR extracellular region.[9] Also, it should be noted that certain RTKs, such as the insulin and insulin-like growth factor receptors, form inactive dimers that are triggered in the presence of their ligands through conformational changes.[9]

Among the different RTKs, the tyrosine kinase is the most conserved domain, and its integrity is absolutely required for receptor signaling. For example, mutation of a single lysine in the ATP binding site, which blocks the ability of the receptor to phosphorylate tyrosine residues, completely inactivates receptor biological function.[10] In the presence of their ligands, the conformational changes in the activated receptors overcome *cis*-autoinhibitory interactions in the kinase, the juxtamembrane, and the carboxy-terminal domains, which allow *trans*-phosphorylation on tyrosine residues that serve as docking sites for different adaptor and second messenger proteins.[9]

The juxtamembrane sequence that separates the transmembrane and cytoplasmic domains is not well conserved among different families of receptors. However, juxtamembrane sequences are highly similar among members of the same family, and studies indicate that this stretch plays a role in the modulation of receptor function. For example, addition of PDGF to many types of cells causes a rapid decrease in high-affinity binding of EGF to its receptor, which is mediated by the phosphorylation of the EGFR juxtamembrane domain induced by PDGF-triggered protein kinase C (PKC), a process termed *receptor transmodulation*.[11] The carboxy-terminal domain of the receptor is thought to play an important role in the regulation of kinase activity. This region typically contains several tyrosine residues, which are autophosphorylated by the activated kinase. In fact, the receptor itself is often the major tyrosine phosphorylated species observed following ligand stimulation. Tyrosine phosphorylation of the carboxy-terminal domain can modulate kinase catalytic activity and/or the ability of the kinase to interact with substrates. Thus, mutations that alter individual tyrosine sites or deletions of this domain have the effect of attenuating kinase function.[9,11]

RTKs and Cancer

The constitutive expression of a growth factor and its specific receptor by the same cell may be sufficient to establish a so-called autocrine loop that contributes to tumor progression. Autocrine transforming interactions have been identified in a number of human malignancies. At least one PDGF chain and one of its receptors have been detected in a high fraction of sarcomas and in glial-derived neoplasms.[12-14] Growth factors also contribute to tumor progression by a paracrine mode. For example, continuous stimulation by growth factors in paracrine as well as autocrine modes during chronic tissue damage and repair associated with cirrhosis and inflammatory bowel disease may predispose to tumors.[15] Some tumor cells produce angiogenic growth factors such as the vascular endothelial growth factors (VEGFs). Such growth factors cause paracrine stimulation of endothelial cells, inducing neoangiogenesis and lymphangiogenesis, which contribute to tumor progression.[16]

RTKs are frequently targets of oncogenic alterations, which create a constitutively activated receptor, independent of the presence of ligand. This was initially demonstrated with retroviral oncogenes, v-erbB and v-fms, encoding activated forms of the EGF and CSF-1 receptor, respectively.[6,17] Alterations affecting a large number of RTKs have been implicated in human malignancies. One mechanism involves the amplification or overexpression of a normal receptor. Examples include EGFR, ERBB-2, and MET (see reviews[18,19]). In some human tumors, deletions within the external domain of the EGFR receptor or mutations in its tyrosine kinase domain are associated with its constitutive activation.[19] The *RET* gene is activated by rearrangement, as a somatic event, in about one third of papillary thyroid

carcinomas. Germline mutations affecting the cysteine residues in the extracellular region, resulting in constitutive dimerization of these receptors caused by the formation of intermolecular cysteine bridges, are responsible for multiple endocrine neoplasia (MEN) 2A and for the familial medullary thyroid carcinoma syndrome. In contrast, point mutations in RET kinase domain, such as those involving codons V804, Y806, A883, and M918, are responsible for MEN 2B.[20] These mutations have been shown to upregulate RET catalytic function, resulting in its genetic transmission as an oncogene. MET is overexpressed and/or mutationally activated in a variety of human tumors, including hepatocellular, gastric, and colorectal carcinomas, and activating mutations of *MET* have been associated with the metastatic progression of head and neck cancers.[18] A direct role of MET in hereditary papillary renal carcinoma has also been established.[21] This hereditary disease is characterized by multiple, bilateral renal papillary tumors, in which mutations activate constitutive kinase activity and transforming properties. Somatic mutations in *MET* have also been detected in some sporadic renal papillary tumors.[21] Several other receptors, including PDGF-β, TrkA, TrkC, and anaplastic lymphoma kinase (ALK) have been shown to be oncogenically activated in human malignancies by gene rearrangements that lead to fusion products containing the activated TK domain.[22-25]

Signaling Pathways of Tyrosine Kinase Receptors

Knowledge of the cascade of biochemical events triggered by ligand stimulation of tyrosine kinase receptors has increased rapidly in recent years and provides further evidence of the importance of these signaling pathways in cancer. The PDGF system has served as the prototype for identification of the components of these systems. Certain molecules become physically associated and/or phosphorylated by the activated PDGF receptor kinase. Those identified to date include phospholipase C (PLC)-γ,[26] phosphatidylinositol-3′-kinase (PI-3K) regulatory subunit (p85),[27] Nck,[28] the phosphatase SHP-2,[29] Grb2,[30] Crk,[31] ras p21 guanosine triphosphatase (GTPase)-activating protein (GAP),[32,33] and src and src-like tyrosine kinases.[34] PLC-γ is one of several PLC isoforms and is involved in the generation of two important second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG), which cause release of stored intracellular calcium and activation of PKC, respectively.[35,36] PKC participates in cell proliferation, survival, and migration. Although different isoforms can play distinct and sometimes opposite roles in such processes, evidence for the involvement of PKC in tumorigenesis and cancer progression is substantial.[36]

PI-3K phosphorylates the inositol ring in PI in the 3′ position and becomes physically associated with a number of activated tyrosine kinases.[37,38] This protein contains an 85-kDA regulatory subunit, which is tyrosine phosphorylated, and a 110-kDa catalytic subunit. PI3-K appears to play a major role in cell survival signaling, as discussed later (see Figure 2-1).

RAS

Ras small GTP-binding proteins are a major point of convergence in receptor tyrosine kinase signaling and are an important component of the cellular machinery necessary to transduce extracellular signals (see review[39]). These membrane-bound intracellular signaling molecules mediate a wide variety of cellular functions, including proliferation, differentiation, and survival. The Ras family of GTPases contains 39 proteins encoded by 36 genes in the human genome; it includes H-, N-, and K-Ras, R-Ras, Rap1 (A and B), TC21, and Rheb1/2.[39] Ras proteins are synthesized in the cytosol and become associated with the inner leaflet of the plasma membrane via posttranslational modifications, including a form of fatty acid lipidation, isoprenylation, on Cys-186. The carboxy-terminal CAAX box (Cys, two aliphatic amino acids, followed by any residue) is an essential motif required for Ras function, as it targets the unprocessed protein for this essential modification.

Ras proteins act as molecular switches alternating from an inactive guanosine diphosphate (GDP)-bound state to an active GTP-bound state. The paradigm for Ras activation involves the recruitment of a guanine nucleotide exchange factor (GNEF) to the membrane in response to growth factor binding and subsequent activation of a receptor tyrosine kinase.[39] GNEFs promote the release of GDP from the Ras catalytic pocket, and the relative abundance of intracellular GTP as compared to GDP ensures preferential binding of GTP (Figure 2-2). The best example of a Ras GNEF is SOS (son of sevenless), which is brought to the membrane by its stable association with the adaptor protein Grb2 (see Figure 2-1[40]). Grb2 contains an src-homology 2 domain (SH2), which binds to a specific motif containing phosphorylated tyrosine residues on several RTKs, including the PDGFR and the EGFR. Grb2 also has two SH3 domains that mediate its binding to SOS via a carboxy-terminal proline-rich region. Alternatively, another adaptor protein, Shc, can bind to the cytoplasmic tail of the receptor through its SH2 domain, resulting in its phosphorylation on tyrosine and subsequently binding Grb2.[41] The exact sequence of binding of adaptors depends on the receptor and cell type. Once SOS is translocated to the membrane, it can promote the release of GDP from Ras, allowing GTP, which is present in excess in the intracellular environment, to

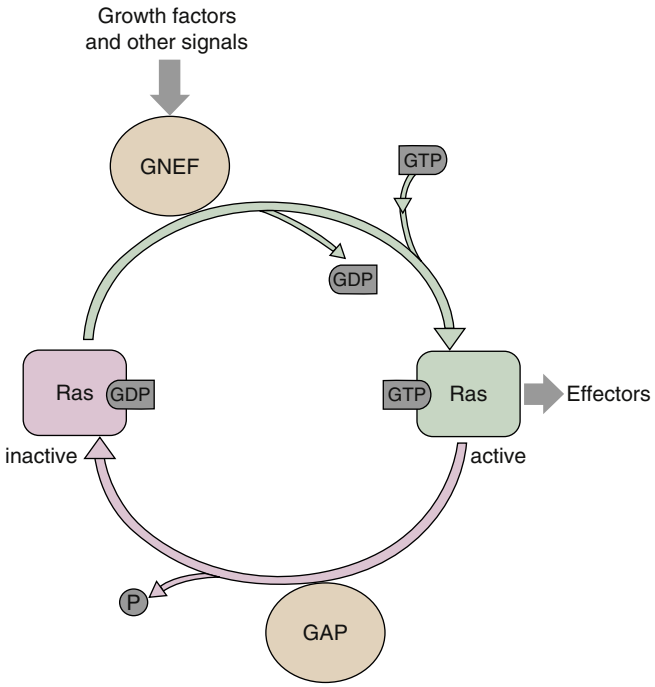


Figure 2-2 Activation of Ras GTPase

bind and ultimately lead to Ras activation. Although Ras is a GTPase, its intrinsic GTPase activity is actually quite inefficient and requires additional proteins known as GTPase activating proteins (GAPs) to promote GTP hydrolysis (see Figure 2-2). GAPs can accelerate GTP hydrolysis by several orders of magnitude and are, thus, negative regulators of Ras functions.[39,42] The mechanism by which GAP accelerates the GTPase reaction is complex and not completely understood. Currently, several GAPs for Ras have been identified, including p120 GAP and NF1-GAP/neurofibromin, as well as GAPs with preferential activity on related proteins such as Rap.[42] Of particular interest is NF1, as it is found to be frequently inactivated by mutation in patients with the familial tumor syndrome neurofibromatosis type 1.

Ras Functions

Ras appears to have a multitude of functions that differ depending on factors such as cell type and extracellular environment. It is paradoxical that a single gene can cause cell cycle entry and DNA synthesis in one type of cell, such as fibroblasts, and terminal differentiation in others, such as PC12.[43,44] In other cell types such as myoblasts, activated Ras seems to oppose cell cycle withdrawal and differentiation into myotubes and downregulates expression of muscle-specific mRNA transcripts.[45] In addition, Ras has been demonstrated to promote cell survival in some cell types such as those of hematopoietic lineages on cytokine withdrawal, and PC12 cells and primary sympathetic neurons on removal of NGF or other trophic factors.[46,47] Although Ras mediates such important cellular processes as proliferation, survival, and differentiation, the exact contribution of H-, N-, and K- isoforms is not clear: targeted knockouts to H- and N-Ras genes resulted in mice that did not exhibit an abnormal phenotype, whereas a K-Ras knockout is an embryonic lethal and exhibits liver and hematopoietic defects.[48,49] Of note, H-Ras knockin into the K-Ras locus does not perturb mouse embryonic development, implying that the phenotype of K-Ras$^{-/-}$ mice is probably due to the different expression pattern of the other Ras isoforms in the embryo, rather than their incapacity to compensate for K-Ras function.[39]

Ras and Cancer

The initial evidence for Ras involvement in cancer came from the discovery of transforming retroviruses, Harvey and Kirsten sarcoma viruses, which contained H- and K-ras cellular derived oncogenes. The first human oncogenes were identified by transfecting genomic DNA from human tumor cell lines into NIH3T3 mouse fibroblasts and isolating the DNA fragments from the transformed foci. These were shown to be the human homologues of the viral ras genes.[43] Subsequent studies have shown that Ras is oncogenically activated by mutations in about 30% of all human tumors, and in some cancers, such as pancreatic carcinoma, the frequency is as high as 60%.[50]

Mutations in human tumors have been found at residues 12, 13, and 61, with positions 12 and 61 being the most common.[50] These mutations decrease the intrinsic rate of GTP hydrolysis by Ras, as well as make the molecule significantly less sensitive to GAP-stimulated GTP hydrolysis. Thus, the outcome is a molecule that is predominantly GTP bound, constitutively active, and able to activate downstream pathways in the absence of growth-factor stimulation. Oncogenic Ras is capable of transforming immortalized rodent fibroblasts or epithelial cells.[43] Ras transformed cells appear refractile and spindle shaped, have disorganized actin filaments, and have a decreased affinity for the substratum. They can proliferate in the absence of adhesion (anchorage independence) or in the presence of low serum concentration. Such cells exhibit a loss of contact inhibition and grow to high saturation density. Of note, however, Ras alone is unable to transform primary mouse or human fibroblasts and instead causes such cells to undergo permanent growth arrest, also termed *replicative senescence*, characteristic of primary cells passed for multiple generations in culture. This senescence response appears to be dependent on the acute upregulation of p16(INK4A) induced by high levels of Ras expression, and it can be overcome by the loss of function of p16(INK4A) and p53 tumor suppressors (see Chapter 3), which may help to explain the selective pressure for inactivation of these genes in tumors containing Ras oncogenic mutations.[39,51]

Not only is Ras itself mutated or overexpressed in cancer, but there are examples of Ras regulatory proteins, which can be affected as well. The best example is NF1, a Ras GAP mentioned earlier. Hereditary transmission of a defective NF1 allele predisposes an individual to a genetic disease called *neurofibromatosis type 1* or *von Recklinghausen's neurofibromatosis*.[52] Somatic mutations result in the inactivation of the second allele, leading to neoplastic development. Neurofibromatosis can manifest itself with the occurrence of multiple benign neurofibromas, as well as a high risk for malignancies of neural crest origin. In cells with defective NF1, cellular Ras accumulates in its GTP-bound state and thus is more active.[52] Other examples of Ras modulators involved in human hereditary diseases are the SH-2–containing tyrosine phosphatase SHP2 and the GNEF SOS1, whose mutations have been detected in patients affected by Noonan syndrome.[39,50]

Signaling Downstream of RAS

Ras>Raf>Map Kinase Cascade

The most wellstudied effector of Ras is the serine/threonine kinase Raf (see Figure 2-1). Raf has been shown to bind to Ras and in many cases has been demonstrated to be indispensable for Ras functions such as cellular transformation.[53] In fact, activated Raf or v-Raf, a truncated form of c-Raf, was initially isolated as a retroviral oncogene. There are three known mammalian Raf isoforms designated A-, B-,and C-Raf (also known as Raf-1) (for review, see Ref. 54). C-Raf is ubiquitous in its tissue expression, whereas A-Raf and B-Raf expression is more restricted. Ras-mediated activation of Raf requires binding to two regions of this cytoplasmic kinase, both of which are located at the amino terminus. In the active state, Raf proteins form homo- or heterodimers, a process that is enhanced by Ras and inhibited by the extracellular signal regulated kinase (ERK) in a negative feedback loop.[55] Several phosphorylation events on both serine/threonine and tyrosine residues play a role in the full activation of Raf,[54,55] and there are major differences in certain phosphorylation sites between A/C-Raf and B-Raf. Also, B-Raf displays the highest basal kinase activity, which may explain why B-Raf, but not A/C-Raf, is frequently mutated in certain types of cancer.[55]

Once activated, Raf can phosphorylate MEK (mitogen/extracellular-signal regulated kinase kinase), a dual-specificity kinase, on Ser_{218} and Ser_{222}, leading to its activation (see review[56] and Figure 2-1). Partial activation can be seen by phosphorylation on only one serine. There are two isoforms of MEK, designated MEK1 and 2, both of which are expressed ubiquitously with an approximate sequence identity of 80%. MEK, once activated, can in turn activate mitogen-activated protein (MAP) kinase (also designated ERK).[56] Activation occurs via tandem phosphorylations on both threonine and tyrosine (Thr_{183}-Glu-Tyr_{185}) with the phosphorylation on tyrosine occurring first. There are two ERK isoforms (1 and 2), ubiquitously expressed and with very similar sequences. These proteins, 44 and 42 kDa, respectively, translocate to the nucleus, where they can activate a variety of proteins through phosphorylation on serine or threonine. For example, ERK can phosphorylate several of the members of the Ets family of transcription factors, and phosphorylation of Ets-1 by ERK dramatically increases c-fos transcription. ERK can also activate a variety of protein kinases via phosphorylation. For example, p90 RSK is a serine/threonine kinase, which has a role in protein translation and has been shown to be a substrate for ERK.[57]

In addition to positive regulation of the MAP kinase pathway by phosphorylation, there are negative regulatory mechanisms that serve to attenuate activation of this cascade. A principal mode of this negative regulation is through a variety of phosphatases, a majority of which are dual specific, meaning they can dephosphorylate both serine/threonine and tyrosine residues. This is consistent with knowledge that ERK must be phosphorylated on both threonine and tyrosine to achieve maximal activation.[58]

Functions of the MAP Kinase Pathway

As mentioned previously, the MAP kinase cascade mediates many Ras downstream functions (see Figure 2-1). ERK activation can lead to increased DNA synthesis and cell proliferation. In fact, activated forms of Ras, Raf, and MEK induce expression of *cyclin D1*, which plays a major role in early cell cycle progression.[59] Dominant negative mutants of members of this cascade can also block this induction in response to growth factor stimulation. In fact, *cyclin D1* is one of the most frequently amplified genes in cancer,[60] and this locus is almost invariably translocated in certain lymphomas, associated with cyclin D1 overexpression.[59]

Raf/Mek/MapK and Cancer

Davies and colleagues[61] identified B-Raf mutations in around 60% of human melanoma cell lines and primary tumors. Of note, these mutations, which in most cases involve the substitution of glutamate for valine 600 (V600E), were not consistent with mutations typically induced by UV. Lower frequencies of analogous mutations were observed in colon carcinoma and small-cell lung cancer (SCLC).[61] These mutations were further shown to oncogenically activate B-Raf, and recently mutant B-Raf has been successfully targeted in melanoma, as discussed later. In contrast to Ras and B-Raf, mutations of other Raf genes, MEK, or ERK are rare in cancer, although rearrangements causing aberrant activation of C-Raf have been recently reported in human prostate cancer.[62]

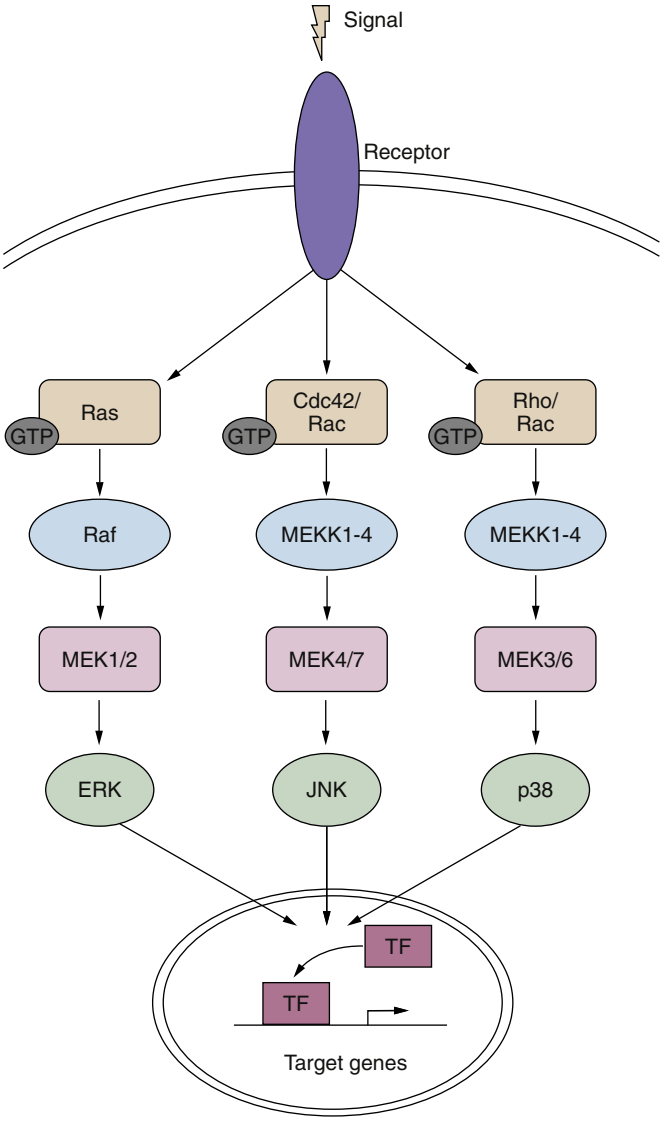


Figure 2-3 MAP kinase pathways in cancer Activation of the three MAP kinase cascades: ERK, JNK, and P38.

Other MAP Kinases

In addition to the ERKs, there are other MAP kinases belonging to distinct MAPK cascades with both different upstream activators and downstream effectors (Figure 2-3). The c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAP kinase have been demonstrated to modulate cellular responses to a wide variety of extracellular stimuli including mitogens, inflammatory cytokines, and UV irradiation (see review[63]). There are three JNK genes, each with several alternatively spliced transcripts. In most cell types examined, including fibroblasts, epithelial cells, and neuronal-like PC12 cells, sustained activation of JNK/SAPK has been reported to induce programmed cell death, whereas transient activation can promote cell survival.[63] There is evidence for some redundancy among these three genes, as each of the single knockouts as well as the JNK1/JNK3$^{-/-}$ and JNK2/JNK3$^{-/-}$ double knockouts is viable, but mice lacking both JNK1 and JNK2 are embryonic lethal. However, it has been demonstrated that the three JNK isoforms can also exhibit distinct properties, for example, in their capacity to interact with the oncogene Jun.[63]

In contrast to its ability to activate the MAPK/ERK cascade, H-ras only minimally perturbs JNK/SAPK. However, overexpression of the constitutively activated mutants of the small G-proteins, Rac and Cdc42, leads to the robust stimulation of JNK/SAPK activity. The pathways leading to JNK activation mirror those seen for ERK. Thus, a variety of MAP kinase kinases (MKK) can phosphorylate the various JNK isoforms.[56,63]

As with the ERKs, JNK activation results in phosphorylation of certain transcription factors and increases their transcriptional activity at promoters containing response elements for these factors.[63] Some of the transcription factors activated by ERK or JNK were initially discovered as retroviral oncogenes in mice and chickens, respectively. The FBJ and FBR murine viruses contain the *Fos* sequence under the viral LTR promoter and exhibit changes in regulatory phosphorylation sites that make them more active than the proto-oncogene,[64] and Jun was identified as an avian retrovirus.[65] Overexpression of Fos can cause transformation of cells as well.[66] Jun and Fos belong to the AP-1 family of transcription factors. In response to various stimuli, including UV irradiation, environmental stresses, and PKC activation, Jun homodimers and Jun-Fos heterodimers bind to AP-1 target sequences such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive elements.[67]

C-Myc

The Myc family includes four transcription factors, c-Myc, N-Myc, L-Myc, and S-Myc, involved in the control of cell growth, differentiation, and apoptosis.[68,69] Myc proteins form heterodimers with another transcription factor, Max, through a basic-region/helix-loop-helix/leucine-zipper domain and bind a specific DNA consensus sequence called E-box to activate the transcription of target genes. In the absence of Myc, Max forms a complex with Mad/Mnt proteins and acts as a repressor of Myc transcriptional targets. A large number of genes are regulated by Myc, including *p21(CIP1)*, *cyclin D1/D2*, *E2F2*, and the microRNA *miR-17-92* cluster. Because the E-box sequence is very frequent in the human genome, more sophisticated approaches, such as genome-wide chromatin immunoprecipitation, have been used to identify authentic and physiologically relevant Myc target genes.[69] Enhanced expression of Myc proteins is estimated to occur in up to 70% of human malignancies. However, the deregulated expression of these proteins is

not sufficient to induce cell transformation, implying that additional genetic events are required. One such event is the activation of the Ras pathway, which affects Myc factors at different levels, including posttranslational stabilization and inhibition of the antagonizing transcription factors FOXO. Also, because high levels of Myc can trigger apoptosis, tumor cells may need for their survival concomitant oncogenic events, such as Ras activation or loss of ARF or p53 tumor suppressors.[68,69] In lymphoid cancers, c-Myc is often found in translocations adjacent to a strong promoter, such as that of the immunoglobulin genes. In other cancers such as breast and lung carcinoma, the genomic locus encoding c-Myc is amplified. c-Myc overexpression can also result from the aberrant activation of upstream pathways, such as in the case of Wnt signaling discussed later.[68,69] Gene amplification is also the mechanism responsible for the increased expression of N-Myc commonly observed in certain cancers, including retinoblastoma, glioblastoma, and medulloblastoma. Of note, N-Myc overexpression in neuroblastoma strongly correlates with an advanced clinical stage, and it is taken into consideration for the assessment of the treatment of these malignancies.[70]

Oncogenes and Survival Signaling

The regulation of cell survival and cell death is of extreme importance both in the development of an organism and in the physiologic functions of the adult. During development of a multicellular organism, certain cells are eliminated by a process known as apoptosis or programmed cell death and others are permitted to survive. The deregulation of these processes can lead to a variety of malformations resulting in deformities or, in extreme cases, incompatibility with life. In adulthood, regulation of cell survival is equally important for proper homeostasis. Damaged cells must be removed, and terminally differentiated cells must be sustained. If this fails to occur, mutations leading to cancer may accumulate. Pro-apoptotic and anti-apoptotic proteins regulate these processes, and many of the oncogenes already discussed modulate cell survival in a positive fashion. Thus, oncogenes can influence proliferation, cell survival, or both, contributing to cellular transformation in a cooperative fashion.

The Bcl-2 Family

The Bcl-2 family of proteins consists of more than 15 members, which can be subdivided into three classes based on functions and the number of Bcl-2 homology (BH) domains present.[71,72] The anti-apoptotic members, including

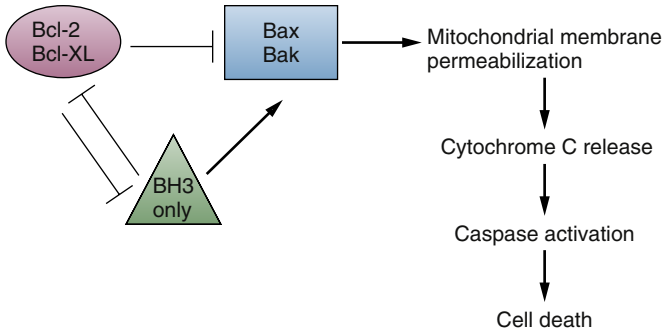


Figure 2-4 Bcl-2 family member interactions regulate cell death

Bcl-2, Mcl-1, and Bcl-XL, contain four BH domains (BH1 to BH4). The pro-apoptotic BCL-2 factors are divided into the effector proteins, such as BAX and BAK, which also contain four BH domains, and the "BH3 only" pro-apoptotic proteins, such as BID and BIM, which include only the BH3 domain. Proteins in all three classes have the ability to form either homo- or heterodimers with one another and play distinct roles in regulating mitochondrial membrane permeabilization[71,72] (Figure 2-4).

The involvement of Bcl-2 and cancer has been firmly established. Not only was the gene cloned as a translocation from a lymphoid tumor, but mice expressing a *Bcl-2*-immunoglobin "mini-gene" that mimicked the translocation seen in human cancers showed follicular hyperplasia that progressed to lymphoma. The *Bcl-2* genomic locus has been found to be translocated in several tumor types, including follicular lymphomas and chronic lymphocytic leukemia, and other oncogenes, such as Ras, can stimulate Bcl-2 expression.[72] The *Mcl-1* and *Bcl-X* genes are frequently amplified in tumors,[60] and overexpression of anti-apoptotic family members as well as downregulation or inactivation of pro-apoptotic proteins has been observed in several human cancers.[72]

PI3K-Dependent Pathways

PI3K is a lipid kinase that catalyzes the transfer of the γ-phosphate from ATP to the D3 position of phosphoinositide (PtdIns), generating PtdIns3P, PtdIns(3,4)P2, and PtdIns(3,4,5)P373 (see Figure 2-1). These lipids can act in a variety of cascades. PI3K activation has been demonstrated to play an important role in cell survival signaling in a number of cell types. There are three classes of PI3Ks, which exhibit variability with respect to their method of activation or their preferred lipid substrate.

The class I of PI3K, and in particular the subclass I_A, is the most relevant for cancer and the best characterized. It consists of two subunits encoded by two distinct loci, a regulatory and a catalytic subunit.[37] The regulatory subunit is a 50- to 85-kDa protein that is tightly associated with the

p110 catalytic subunit. The classical mode of PI3K activation involves its binding to the phosphorylated tyrosine residues of receptor tyrosine kinases via the two SH2 domains of p85. This results in the recruitment of the p85-p110 heterodimer to its substrate at the cell membrane and in a conformational change that relieves the basal inhibition of p110 catalytic activity.[37] In addition, it has been demonstrated that PI3K can be activated independently of receptor binding by the small G-protein Ras[37,74] (see Figure 2-1). Furthermore, the γ isoform of PI3K is activated by heterotrimeric G-proteins.[37] Thus it is clear that PI3K can be activated in response to a wide variety of upstream signals.

There are several known downstream effectors of PI3K. These include Rac, p70(s6k), certain isoforms of PKC, and, most relevant to the discussion of cell survival, Akt/PKB.[37,73] Akt has been shown to be responsible for PI3K-dependent cell survival signaling and is the cellular homologue of the viral oncogene v-Akt. The three human homologues identified encode 57-kDa serine/threonine kinases that contain an amino-terminal pleckstrin homology domain, which binds to the activated PtdIns products of PI3K. These lipids are believed to mediate the localization of this cytoplasmic protein to the plasma membrane. In addition, phosphorylation of Akt on two residues, a serine and a threonine, is required for full activation. These events are catalyzed by two different kinases, one of which, PDK1 (PtdIns(3,4,5)P3 dependent kinase) specifically phosphorylates Thr^{308}; the other, the mTOR (mammalian target of rapamycin) complex 2, phosphorylates Ser^{473}.[37,74] Once activated, Akt directly phosphorylates different proteins, such as BAD, forkhead box O transcription factors, glycogen synthase kinase 3 (GSK3), and tuberous sclerosis 2 (mTOR signaling), thus regulating various processes, including cell survival, proliferation, and protein synthesis[74] (see Figure 2-1).

The striking anti-apoptotic effect of both PI3K and its downstream effector Akt, as well as the fact that these two genes were initially found as transforming viral oncogenes, suggested that these two genes might also be involved in human cancer. Indeed, a myristoylated constitutively active PI3K can cause cellular transformation in chicken embryo fibroblasts.[75] The gene encoding the p110α catalytic subunit is frequently mutated in certain common epithelial cancers, such as breast, colon, and prostate. One class of mutation, which promotes constitutive signaling, involves the kinase domain near the activation loop. The second class of *p110α* mutations is predicted to abrogate the inhibitory intermolecular interaction between p85 and p110. Consistent with this model, mutations affecting the p85 subunit have recently been identified in glioblastomas and in colon and ovarian cancers.[74] Activating mutations of the three Akt isoforms have been also described, although they appear to be far less common than those involving the *p110α* locus. In addition, certain cancers can harbor amplifications of p110α or Akt subunits.[74]

Further evidence of the involvement of the PI3K/Akt pathway in cancer stems from the discovery of PTEN, one of the most frequently inactivated tumor suppressor genes in different types of tumors, including glial and endometrial tumors, as well as melanoma, prostate, renal, and small-cell lung carcinomas (for a review, see Ref. 76). Germline mutations at the *PTEN* locus cause inherited cancer syndromes such as Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome. PTEN dephosphorylates the 3 position of phosphatidylinositol, thus directly opposing PI3K activity[76] (see Figure 2-1).

Cytokine Receptor Signaling

A large number of cytokines, hormones, and growth factors have been shown to activate a class of receptors that lack significant sequence similarity to the RTKs and are grouped under the definition of cytokine receptors.[77] They share a common structural motif in their extracellular domains, including conserved cysteine residues, and lack intrinsic enzymatic activity. The cytokine receptors either homodimerize on ligand binding (receptors for growth hormone, prolactin, erythropoietin, and thrombopoietin) or are composed of two distinct subunits that heterodimerize in response to ligand interaction. This latter group of receptors is composed of a ligand-specific chain and a common chain shared by different cytokines. This includes the receptors for interleukin-6 (IL-6), IL-11, oncostatin M, LIF, cardiotrophin-1, and ciliary neurotrophic factors, all sharing a common chain called gp130; the receptors for IL-3, IL-5, and GM-CSF that share the β common chain; and the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 that share the γ common chain.[78]

The Janus kinases (JAKs) originally identified as signaling molecules in the interferon pathway are essential transducers of the signal originating from cytokine receptors.[79] Four mammalian JAKS have been identified, JAK-1, JAK-2, JAK-3, and Tyk-2. These kinases are associated with the receptors and contain both a catalytic domain and a pseudokinase domain that is involved in the inhibition of JAK activity in the absence of the ligands. On ligand binding, the activated JAKs cause phosphorylation of the receptor and of molecules containing either a phosphotyrosine-binding or an SH2 domain. These molecules comprise the signal transducers and activators of transcription (STATs).

Seven mammalian STATs have been identified (STAT-1, STAT-2, STAT-3, STAT-4, STAT5a, STAT5b, and STAT-6), and differential splicing increases the number of these molecules. In addition to the SH2 domain, their structure is characterized by a DNA-binding domain and several protein-protein interaction domains. After becoming

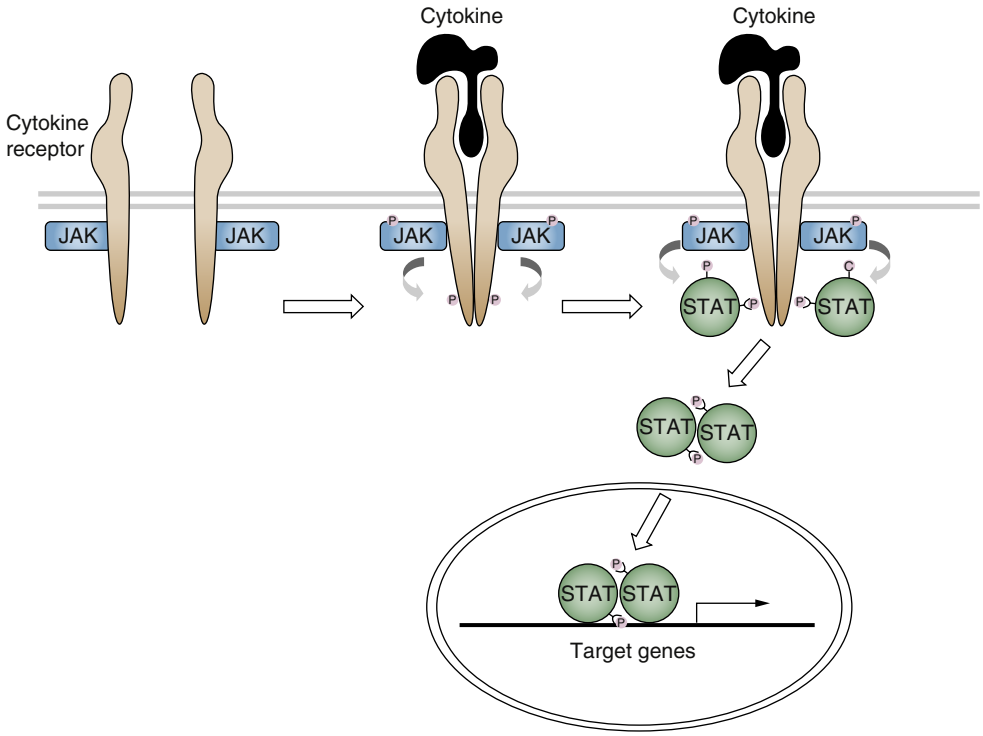


Figure 2-5 Cytokine receptor signaling The binding of cytokines to their receptors activates the JAK/STAT pathway through a series of phosphorylations.

phosphorylated on tyrosine, STATs form homo- or heterodimers through their SH2 domain and translocate to the nucleus, where they activate target genes (Figure 2-5[80,81]). In addition to the cytokine receptors, the JAK/STAT pathway has been shown to transduce signals from a number of tyrosine kinase receptors, including those for EGF, PDGF, and CSF-1.[80]

Several components of the cytokine receptor signaling pathways have been implicated in uncontrolled cell proliferation and cancer. The myeloproliferative virus (MPLV), an acute transforming retrovirus, contains an oncogene called v-mpl, which is a truncated version of a member of the hematopoietin receptor family c-mpl whose ligand has been identified as the thrombopoietin. Ectopic expression of c-mpl induces a lethal myeloproliferative disease in mice,[82] and activating mutations have been identified in myelofibrosis with myeloid metaplasia in humans.[83] The first evidence of involvement of the JAKs/STATs in naturally occurring cancer was the finding of an activating mutation in the *Drosophila* Hop kinase, a member of the JAK family, that caused a leukemia-like phenotype.[84] Stronger evidence implicating the JAKs in a human cancer came from the identification of a chromosomal translocation in a human leukemia resulting in the constitutively activated fusion protein TEL-JAK2.[85] In 2005, various groups identified a mutation in the *JAK2* gene in a large proportion of patients with different types of myeloproliferative neoplasms, including polycythemia vera (PV) and essential thrombocythemia. This mutation involves the substitution of a phenylalanine for valine 617 (V617F) and is believed to disrupt the autoinhibitory activity of the pseudokinase domain, resulting in cytokine hypersensitivity and cytokine-independent growth.[81] It has been shown that in hematopoietic cells containing JAK2(V617F), signaling pathways involved in cell proliferation and survival are activated, including STAT, MAPK, and PI3K-AKT.[81] More recently, activating mutations in JAK2 exon 12, which encodes a region near the pseudokinase domain, were identified in JAK2(V617F) negative PV patients.[81] Although activating mutations of STAT3 have been identified only in a particular type of liver tumor,[86] this transcription factor is very frequently activated through various mechanisms in cancer cells. It is thought not only to participate in tumor cell proliferation, survival, and invasion, but also to suppress antitumor immunity and to maintain a procarcinogenic inflammatory microenvironment.[87]

Neurotransmitters

The transmission of signals generated by the reception of chemical and physical stimuli from the external and internal environments is mediated by a large variety of small molecules known as *neurotransmitters*. These molecules include acetylcholine; amino acid derivatives such as epinephrine, norepinephrine, serotonin, and dopamine; and peptides such as the angiotensins, β-endorphin, enkephalins, and

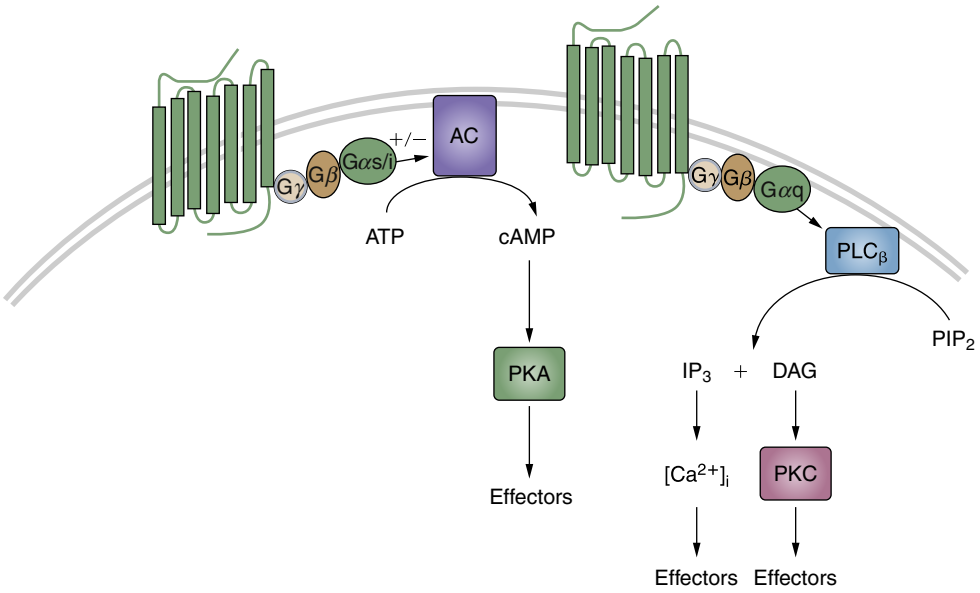


Figure 2-6 G-protein coupled receptors (GPCRs) Diagram showing cyclic adenosine monophosphate (cAMP) and phospholipase C-β (PLC_β) transduction pathways activated by GPCRs.

somatostatin. These ligands can trigger two types of receptors: ion-channel–linked receptors or receptors with seven membrane-spanning domains, which interact with heterotrimeric G proteins composed of α, β, and γ subunits. After binding to their specific ligand, the G-protein coupled receptors (GPCRs) undergo a conformational change, which results in a switch from the inactive GDP-bound G_α to an active GTP-bound state and the dissociation of the $G_{\beta\gamma}$ subunits. Different subfamilies of G_α proteins exist that activate various signaling pathways. For example, $G_{\alpha s}$ and $G_{\alpha i}$, respectively, stimulate or inhibit adenylyl cyclase, provoking an increase (or a decrease) of cyclic AMP levels, which can then activate the protein kinase A (Figure 2-6). The members of another G_α subfamily, $G_{\alpha q}$, activate PLC-β, which catalyzes the cleavage of phosphatidylinositol biphosphate into DAG and IP_3. DAG then stimulates PKC, whereas IP_3 mobilizes the intracellular stocks of calcium (see Figure 2-6). The $G_{\beta\gamma}$ subunits are also implicated in the signaling cascade, by regulating the activity of different effectors, such as phospholipases, ion channels, and various kinases. Of note, GPCR activation can impinge on other transduction pathways, including Rho and Ras GTPases or MAP kinases, while the mechanisms involved are not completely elucidated (for review, see Ref. 88).

The ability of GPCRs to activate various transduction pathways that regulate cell differentiation and proliferation strongly suggests a potential role of these receptors in tumorigenesis. Indeed, activating mutations of the thyroid-stimulating hormone receptor commonly occur in thyroid adenomas and carcinomas, and germline mutations cause familial non-autoimmune hyperthyroidism.[88] Another example is illustrated by studies on two distinct groups within a subset of growth hormone–secreting human pituitary tumors.[89] In one group, $G_{\alpha s}$ was found to be constitutively active, resulting in elevated adenylate cyclase activity and growth hormone levels. This activation was due to point mutations either in a site at which cholera toxin inactivates $G_{\alpha s}$ [Arg 201 → Cys/His] or at a residue equivalent to a GTPase-inhibiting mutation that causes malignant activation of Ras p21 [Gln 227 → Arg]. Because both mutations have the effect of destroying GTPase activity, $G_{\alpha s}$ [designated *gsp*] becomes constitutively activated in a manner analogous to the oncogenic activity of Ras p21. The two mutations are located in regions that are highly conserved among G_α proteins isolated from diverse eukaryotic species, and activating mutations have been identified in some human adrenal, pituitary, and other endocrine tumors.[90] Although mutations in GPCRs and G proteins have been identified in some tumors, the most common mechanisms of GPCR activation in cancer cells are receptor overexpression and autocrine stimulation,[88] as has been shown, for example, for the gastrin-releasing peptide,[91] angiotensin II,[92] and cholecystokinin[93] in pancreas and prostate cancers. Moreover, the receptors for certain neuropeptides and/or neurotransmitters, including acetylcholine, bradykinin, bombesin, and endothelin, can promote the proliferation and invasion of different types of tumor cells through cross talk with the EGFR or the insulin-like growth factor 1 receptor.[94] Of note, the specific expression of neurotransmitters and their cognate receptors in tumors arising from the endocrine system make this signaling a promising target for cancer diagnosis and therapy.[95,96] Finally, it is worth mentioning that other families of ligands triggering GPCRs play important roles in tumor initiation and progression. This is the case, for example, with the prostaglandins, which mediate chronic inflammation, increasing the risk of tumors, and the chemokines, crucially involved in cancer metastasis.[88,94]

Wnt Signaling

Wnts comprise a highly conserved multimember ligand family and play important roles in a variety of developmental processes, including patterning and cell fate determination.[97,98] In different adult tissues, Wnts also play important roles in stem/progenitor cell maintenance and differention.[98-100] Wnts bind to two co-receptors, the seven-transmembrane Frizzled and single-membrane–spanning low-density receptor-related protein 5/6 (LRP5/6), resulting in the phosphorylation of the LRP5/6 intracellular domain.[101] The phosphorylated LRP5/6 provokes the inhibition of a multiprotein complex, referred to as the *destruction complex*, which includes axin, GSK3, casein kinase 1 (CK1), and adenomatous polyposis coli (APC). In the absence of Wnt ligands, this complex induces the sequential phosphorylation of β-catenin by CK1 and GSK3, which provokes its degradation through the ubiquitination pathway. Thus, Wnt-induced inhibition of the destruction complex blocks β-catenin degradation and causes its accumulation in the cytoplasm in an uncomplexed form. The latter is then translocated to the nucleus, where it binds to transcription factors belonging to the T-cell factor/lymphoid enhancer factor family and activates the expression of Wnt target genes, including the proto-oncogene *c-myc* and *cyclin D1* (Figure 2-7[100]).

The prototype *Wnt* gene was originally identified as a cellular gene activated by integration of the mouse mammary tumor virus.[102] Later studies indicated that targeted expression of certain Wnts in transgenic mice caused mammary gland hyperplasia, and several Wnt genes exhibit the ability to transform various epithelial[103] and fibroblast murine cell lines.[104] Recent evidence indicates that Wnt signaling is constitutively activated through an autocrine mechanism in different types of human cancers, including breast cancer,[105] non–small-cell lung cancer,[106] and sarcoma.[107] More commonly, specific downstream components of the Wnt pathway have been implicated in human cancers. Genetic alterations of β-catenin have been identified in human tumors and cancer cell lines, including colon cancer, melanomas, and hepatocellular carcinomas.[97,108] These mutations affect the sites of phosphorylation of β-catenin and result in the inhibition of its degradation, leading to the stabilization of the protein in the cytosolic and/or nuclear compartments.

The *APC* tumor suppressor gene product, which is required for β-catenin phosphorylation and degradation, also regulates the amount of cytosolic β-catenin. Germline mutations in the *APC* gene are responsible for familial adenomatous polyposis (FAP), a dominantly inherited syndrome characterized by the formation of hundreds of colorectal adenomas, some of which inevitably progress to colorectal cancer. Inactivation of the APC gene leading to increased cytosolic β-catenin is also found in

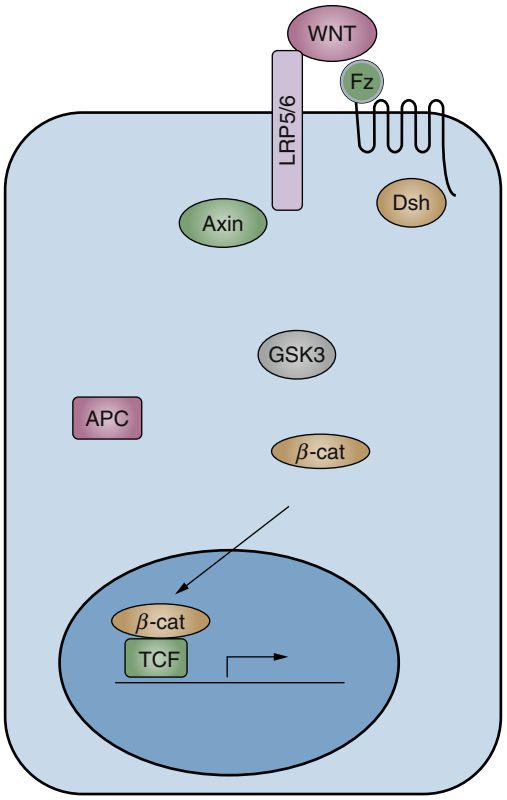


Figure 2-7 Diagram showing the major known components of Wnt signaling in cancer Simplified scheme of canonical Wnt signaling.

80% of sporadic colon cancers, and it represents an early event in tumor progression.[97,108] The major initiating event in the remaining 20% of colon cancer involves mutations in the *β-catenin* gene[97,108] or, as recently uncovered by next-generation sequencing, fusions of the *R-spondin* genes, a family of secreted proteins that enhance Wnt signaling.[109] Of note, these events occur only in those cancer cells with intact APC, implying that activation of this signaling pathway affects almost all colon cancers.

Hedgehog/Patched Signaling

The Hedgehog/Patched signaling pathway was first identified in *Drosophila*, where it plays an important role in a number of developmental processes, including cell fate determination and patterning.[110,111] Although the major core components of the pathway are conserved among species, many differences exist between the fly and vertebrate Hedgehog signaling. In vertebrates, in the absence of the Hedgehog ligands, Sonic, Indian, and Desert Hedgehog, their receptor Patched prevents, through an unknown mechanism, the translocation of the seven-transmembrane domain protein Smoothened to the primary cilium. Under such conditions, the full-length Gli

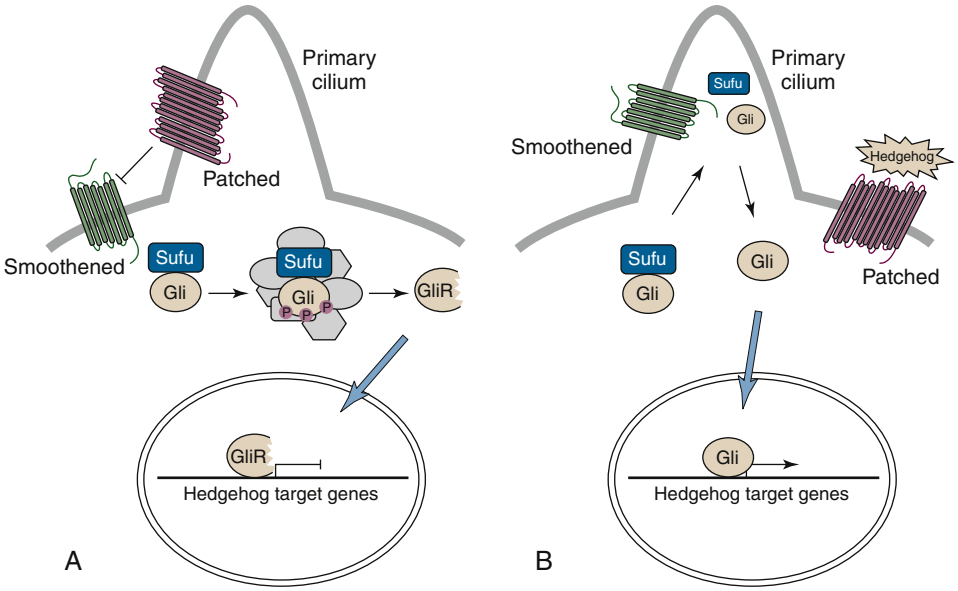


Figure 2-8 Activation of Hedgehog signaling The Hedgehog pathway in vertebrates, in the absence (**A**) or in the presence (**B**) of Hedgehog ligands.

transcription factors bind to suppressor of fused (Sufu) and, following multiple phosphorylation, are cleaved into repressor forms of Gli that inhibit the transcription of Hedgehog target genes. Hedgehog binding to Patched relieves the inhibition of Smoothened, which can then translocate to the primary cilium, where it promotes the disassembly of the Sufu-Gli complex. Gli proteins are shuttled into the nucleus, where they become transcriptionally active, probably after additional posttranslational modifications[112,113] (Figure 2-8).

Several lines of evidence suggest an important role of the Hedgehog pathway in cancer. Mutations in the human homologue of the *Patched* gene have been identified as responsible for the hereditary nevoid basal cell carcinoma (BCC) syndrome,[114] and mutations have also been found in sporadic BCC and medulloblastomas. Loss of Patched would result in the constitutive activation of Smoothened and upregulation of this signaling pathway. Other studies have identified activating missense mutations in *Smoothened* in BCC,[115] further supporting the involvement of this signaling pathway in human cancer. Studies using the Smoothened inhibitor cyclopamine suggested the existence of a Hedgehog autocrine activation loop involved in the growth of different types of cancer. However, it was subsequently demonstrated that Hedgehog ligands do not act cell-autonomously on the tumor cells, but they instead affect the stromal microenvironment through a paracrine mechanism.[116,117]

Implications for Cancer Therapy

The study of signal transduction is crucial to the understanding of the normal cellular processes that govern cellular functioning. Although our knowledge of these intricate events is increasing rapidly, the complexities appear to be growing even more rapidly. What were once believed to be rather simple and linear pathways have now become multidimensional. Signaling pathways converge, diverge, and cross talk so frequently that it is becoming difficult to discuss them as individual pathways. Issues such as cell type specificity, where signaling pathways differ both in how they are activated and in the ultimate outcome, add to the complexities as well. The oncogenes that have been discussed are normally key players in signaling pathways, as illustrated by evidence that constitutive activation of molecules, ranging from receptors to nuclear transcription factors, can cause cellular transformation and/or increased cell survival and are commonly found to be activated in human cancers.

Because many of the signaling pathways involved in cellular transformation by oncogenes have been elucidated, concerted efforts have been made to develop treatment strategies that target these specific signaling molecules or their downstream effectors. Such approaches rely on the idea that tumor cells, despite their complex pattern of mutational events, can become particularly dependent on one or a few signaling pathways for their growth and/or survival, a concept commonly referred to as *oncogenic addiction*.[118] Tremendous strides have been made in developing therapies that target some of the oncogene products discussed in this chapter, and several of these agents have been approved for the treatment of various types of cancer (Table 2-1). Although other approaches are being explored, these new therapeutics consist either of modified monoclonal antibodies, which recognize cell membrane or secreted proteins, such as ERBB2 and VEGF, or of small molecule inhibitors, which target the enzymatic

Table 2-1 Targeted Therapeutics Directed against Oncogene Products

Target	Cancer Drug	Disease
Monoclonal Antibody		
ERBB2	Trastuzumab (Herceptin)	Breast cancer, gastric cancer
ERBB2	Pertuzumab (Perjeta)	Breast cancer
EGFR	Cetuximab (Erbitux)	Colorectal cancer, head and neck cancer
EGFR	Panitumumab (Vectibix)	Colorectal cancer
VEGF	Bevacizumab (Avastin)	Colorectal cancer, NSCLC, RCC, glioblastoma
Fusion Protein		
VEGFA, VEGFB, PGF	Aflibercept (Zaltrap)	Colorectal cancer
Small Molecule		
Abl, PDGFR, c-Kit	Imatinib (Gleevec)	CML, GIST, ALL, dermofibrosarcoma protuberans
Abl, Src	Bosutinib (Bosulif)	CML
Abl, Src	Dasatinib (Sprycel)	CML, ALL
Abl, PDGFR, c-Kit	Nilotinib (Tasigna)	CML
ALK, MET	Crizotinib (Xalkori)	NSCLC
B-Raf (V600E)	Vemurafenib (Zelboraf)	Melanoma
EGFR	Gefitinib (Iressa)	NSCLC
EGFR	Erlotinib (Tarceva)	NSCLC, pancreatic carcinoma
EGFR, ERBB2	Lapatinib (Tykerb)	Breast cancer
mTOR	Everolimus (Afinitor)	RCC, astrocytoma, PNET, breast cancer
mTOR	Temsirolimus (Torisel)	RCC
Smoothened	Vismodegib (Erivedge)	BCC
VEGFR, EGFR, RET	Vandetanib (Caprelsa)	Thyroid cancer
VEGFR, PDGFR, c-Kit	Axitinib (Inlyta)	RCC
VEGFR, PDGFR, c-Kit	Pazopanib (Votrient)	RCC, soft tissue sarcoma
VEGFR, PDGFR, FLT3, c-Kit, Raf, RET	Sorafenib (Nexavar)	RCC, hepatocellular carcinoma
VEGFR, PDGFR, FLT3, c-Kit, RET	Sunitinib (Sutent)	GIST, RCC, PNET

Drugs included in this table have been approved by the Food and Drug Administration (FDA).
ALL, acute lymphoblastic leukemia; *BCC*, basal cell carcinoma; *CML*, chronic myeloid leukemia; *GIST*, gastrointestinal stromal tumor; *NSCLC*, non-small-cell lung carcinoma; *PGF*, placental growth factor; *PNET*, pancreatic neuroendocrine tumor; *RCC*, renal cell carcinoma.

activity of a particular oncogene, generally a kinase, such as Abl, EGFR, or PDGFR. A recent paradigmatic example of such new agents is vemurafenib, an inhibitor of V600E B-Raf approved by the U.S. Food and Drug Administration (FDA) in 2011 for the treatment of unresectable or metastatic melanomas. This small-molecule inhibitor was specifically designed by Plexxikon to selectively block the mutant, but not the wild-type, B-Raf,[119] and it showed a remarkable response rate in melanoma clinical trials.[120] However, as it is often the case with targeted therapy, after an initial response, the tumors relapse and become insensitive to the inhibitor. Several mechanisms have been proposed that allow the tumor cells to bypass the effects of B-Raf(V600E) inhibition, including mutations of Ras and activation of RTKs.[121] Of note, although B-Raf(V600E) occurs in around 10% of colon cancers, this tumor is much less responsive to vemurafenib than is melanoma. It was recently demonstrated that the unresponsiveness of colon cancer is caused by vemurafenib-induced feedback activation of EGFR, a receptor that is highly expressed in colon cancer, but not in melanoma cells.[122] The ability of tumor cells to overcome the inhibition of a particular pathway through activation of alternative mechanisms leading to drug resistance underlines the potential of a therapeutic strategy that simultaneously targets different components of one (e.g., the EGFR and ERBB2 dual inhibitor lapatinib)

or several (e.g., sorafenib, which inhibits VEGFR, PDGFR, FLT3, c-Kit, Raf, and Ret) signaling pathways.

Increased knowledge of oncogene signaling pathways has already led to novel therapeutics, which are in the clinic, and there is great promise that the number of rationally based therapies using such molecules as targets will continue to grow. The astonishing advances in the sequencing of cancer genomes, together with a better understanding of the molecular basis for drug selectivity and acquired resistance, should in the near future allow the design of new therapies based on combinations of targeted agents tailored to the specific genetic alterations of a given tumor.

Key References

1. Pawson T. Dynamic control of signaling by modular adaptor proteins. *Curr Opin Cell Biol*. 2007;19:112-116.
9. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117-1134.
12. Pietras K, Sjoblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. *Cancer Cell*. 2003;3:439-443.
16. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. *Trends Mol Med*. 2011;17:347-362.
18. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer*. 2012;12:89-103.
19. Yarden Y, Pines G. The ERBB network: at last, cancer therapy meets systems biology. *Nat Rev Cancer*. 2012;12:553-563.
20. Runeberg-Roos P, Saarma M. Neurotrophic factor receptor RET: structure, cell biology, and inherited diseases. *Ann Med*. 2007;39:572-580.
21. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*. 2003;4:915-925.
36. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*. 2007;7:281-294.
37. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*. 2006;7:606-619.
38. Vanhaesebroeck B, Stephens L, Hawkins P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol*. 2012;13:195-203.
39. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol*. 2008;9:517-531.
50. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer*. 2007;7:295-308.
54. Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol*. 2004;5:875-885.
55. Maurer G, Tarkowski B, Baccarini M. Raf kinases in cancer-roles and therapeutic opportunities. *Oncogene*. 2011;30:3477-3488.
56. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene*. 2007;26:3279-3290.
57. Anjum R, Blenis J. The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol*. 2008;9:747-758.
58. Bermudez O, Pages G, Gimond C. The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. *Am J Physiol Cell Physiol*. 2010;299:C189-C202.
59. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer*. 2011;11:558-572.
60. Beroukhim R, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
62. Palanisamy N, Ateeq B, Kalyana-Sundaram S, et al. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. *Nat Med*. 2010;16:793-798.
63. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 2009;9:537-549.
68. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6:635-645.
69. Dang CV. MYC on the path to cancer. *Cell*. 2012;149:22-35.
70. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. *Semin Cancer Biol*. 2006;16:318-330.
71. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Mol Cell*. 2010;37:299-310.
72. Strasser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J*. 2011;30:3667-3683.
74. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
76. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol*. 2012;13:283-296.
78. Wang X, Lupardus P, Laporte SL, Garcia KC. Structural biology of shared cytokine receptors. *Annu Rev Immunol*. 2009;27:29-60.
79. Stark GR, Darnell Jr JE. The JAK-STAT pathway at twenty. *Immunity*. 2012;36:503-514.
81. Quintas-Cardama A, Kantarjian H, Cortes J, Verstovsek S. Janus kinase inhibitors for the treatment of myeloproliferative neoplasias and beyond. *Nat Rev Drug Discov*. 2011;10:127-140.
83. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3, e270.
86. Pilati C, Amessou M, Bihl MP, et al. Somatic mutations activating STAT3 in human inflammatory hepatocellular adenomas. *J Exp Med*. 2011;208:1359-1366.
87. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer*. 2009;9:798-809.
88. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer*. 2007;7:79-94.
92. Uemura H, Hasumi H, Ishiguro H, Teranishi J, Miyoshi Y, Kubota Y. Renin-angiotensin system is an important factor in hormone refractory prostate cancer. *Prostate*. 2006;66:822-830.
94. Lappano R, Maggiolini M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov*. 2011;10:47-60.
95. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev*. 2003;24:389-427.
96. van Essen M, Krenning EP, Kam BL, de Jong M, Valkema R, Kwekkeboom DJ. Peptide-receptor radionuclide therapy for endocrine tumors. *Nat Rev Endocrinol*. 2009;5:382-393.
97. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127:469-480.
98. Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev*. 2008;22:2308-2341.
99. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005;434:843-850.
100. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
101. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17:9-26.
105. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA. An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell*. 2004;6:497-506.
106. Akiri G, Cherian MM, Vijayakumar S, Liu G, Bafico A, Aaronson SA. Wnt pathway aberrations including autocrine Wnt activation occur at high frequency in human non-small-cell lung carcinoma. *Oncogene*. 2009;28:2163-2172.
107. Vijayakumar S, Liu G, Rus IA, et al. High-frequency canonical Wnt activation in multiple sarcoma subtypes drives proliferation through a TCF/beta-catenin target gene, CDC25A. *Cancer Cell*. 2011;19:601-612.
108. Polakis P. Drugging Wnt signalling in cancer. *EMBO J*. 2012;31:2737-2746.

109. Seshagiri S, Stawiski EW, Durinck S, et al. Recurrent R-spondin fusions in colon cancer. *Nature*. 2012;488:660-664.
110. Lum L, Beachy PA. The Hedgehog response network: sensors, switches, and routers. *Science*. 2004;304:1755175-1755179.
111. Briscoe J, Therond P. Hedgehog signaling: from the *Drosophila* cuticle to anticancer drugs. *Dev Cell*. 2005;8:143-151.
112. Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat Rev Genet*. 2011;12:393-406.
113. Ryan KE, Chiang C. Hedgehog secretion and signal transduction in vertebrates. *J Biol Chem*. 2012;287:17905-17913.
116. Yauch RL, Gould SE, Scales SJ, et al. A paracrine requirement for hedgehog signalling in cancer. *Nature*. 2008;455:406-410.
117. Ng JM, Curran T. The Hedgehog's tale: developing strategies for targeting cancer. *Nat Rev Cancer*. 2011;11:493-501.
119. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467:596-599.
120. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
121. Poulikakos PI, Rosen N. Mutant BRAF melanomas—dependence and resistance. *Cancer Cell*. 2011;19:11-15.
122. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012;483:100-103.

References

1. Pawson T. Dynamic control of signaling by modular adaptor proteins. *Curr Opin Cell Biol*. 2007;19:112-116.
2. Hunter T, Sefton BM. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A*. 1980;77:1311-1315.
3. McBride OW, Swan DC, Santos E, Barbacid M, Tronick SR, Aaronson SA. Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. *Nature*. 1982;300:773-774.
4. Doolittle RF, Hunkapiller MW, Hood LE, et al. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. *Science*. 1983;221:275-277.
5. Waterfield MD, Scrace GT, Whittle N, et al. Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature*. 1983;304:35-39.
6. Downward J, Yarden Y, Mayes E, et al. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature*. 1984;307:521-527.
7. Carpenter G, Cohen S. Epidermal growth factor. *J Biol Chem*. 1990;265:7709-7712.
8. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature*. 2001;411:355-365.
9. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2010;141:1117-1134.
10. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 1990;61:203-212.
11. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000;103:211-225.
12. Pietras K, Sjoblom T, Rubin K, Heldin CH, Ostman A. PDGF receptors as cancer drug targets. *Cancer Cell*. 2003;3:439-443.
13. Nister M, Libermann TA, Betsholtz C, et al. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. *Cancer Res*. 1988;48:3910-3918.
14. Maxwell M, Naber SP, Wolfe HJ, et al. Coexpression of platelet-derived growth factor (PDGF) and PDGF-receptor genes by primary human astrocytomas may contribute to their development and maintenance. *J Clin Invest*. 1990;86:131-140.
15. Marra F. Chemokines in liver inflammation and fibrosis. *Front Biosci*. 2002;7:d1899-d1914.
16. Saharinen P, Eklund L, Pulkki K, Bono P, Alitalo K. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. *Trends Mol Med*. 2011;17:347-362.
17. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell*. 1985;41:665-676.
18. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. *Nat Rev Cancer*. 2012;12:89-103.
19. Yarden Y, Pines G. The ERBB network: at last, cancer therapy meets systems biology. *Nat Rev Cancer*. 2012;12:553-563.
20. Runeberg-Roos P, Saarma M. Neurotrophic factor receptor RET: structure, cell biology, and inherited diseases. *Ann Med*. 2007;39:572-580.
21. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol*. 2003;4:915-925.
22. Carroll M, Tomasson MH, Barker GF, Golub TR, Gilliland DG. The TEL/platelet-derived growth factor beta receptor (PDGF beta R) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates PDGF beta R kinase-dependent signaling pathways. *Proc Natl Acad Sci U S A*. 1996;93:14845-14850.
23. Greco A, Mariani C, Miranda C, et al. The DNA rearrangement that generates the TRK-T3 oncogene involves a novel gene on chromosome 3 whose product has a potential coiled-coil domain. *Mol Cell Biol*. 1995;15:6118-6127.
24. Greco A, Pierotti MA, Bongarzone I, Pagliardini S, Lanzi C, Della Porta G. TRK-T1 is a novel oncogene formed by the fusion of TPR and TRK genes in human papillary thyroid carcinomas. *Oncogene*. 1992;7:237-242.
25. Scheijen B, Griffin JD. Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease. *Oncogene*. 2002;21:3314-3333.
26. Meisenhelder J, Suh PG, Rhee SG, Hunter T. Phospholipase C-gamma is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell*. 1989;57:1109-1122.
27. Escobedo JA, Navankasattusas S, Kavanaugh WM, Milfay D, Fried VA, Williams LT. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell*. 1991;65:75-82.
28. Nishimura R, Li W, Kashishian A, et al. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol*. 1993;13:6889-6896.
29. Kazlauskas A, Feng GS, Pawson T, Valius M. The 64-kDa protein that associates with the platelet-derived growth factor receptor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. *Proc Natl Acad Sci U S A*. 1993;90:6939-6943.
30. Arvidsson AK, Rupp E, Nånberg E, et al. Tyr-716 in the platelet-derived growth factor beta-receptor kinase insert is involved in GRB2 binding and Ras activation. *Mol Cell Biol*. 1994;14:6715-6726.
31. Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T. Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. *Science*. 1990;250:979-982.
32. Kaplan DR, Morrison DK, Wong G, McCormick F, Williams LTPDGF. beta-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. *Cell*. 1990;61:125-133.
33. Molloy CJ, Bottaro DP, Fleming TP, Marshall MS, Gibbs JB, Aaronson SA. PDGF induction of tyrosine phosphorylation of GTPase activating protein. *Nature*. 1989;342:711-714.
34. Ralston R, Bishop JM. The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor. *Proc Natl Acad Sci U S A*. 1985;82:7845-7849.
35. Berridge MJ, Irvine RF. Inositol phosphates and cell signalling. *Nature*. 1989;341:197-205.
36. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer*. 2007;7:281-294.
37. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet*. 2006;7:606-619.
38. Vanhaesebroeck B, Stephens L, Hawkins P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol*. 2012;13:195-203.
39. Karnoub AE, Weinberg RA. Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol*. 2008;9:517-531.
40. Buday L, Downward J. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*. 1993;73:611-620.
41. Margolis B, Skolnik EY. Activation of Ras by receptor tyrosine kinases. *J Am Soc Nephrol*. 1994;5:1288-1299.
42. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nat Rev Cancer*. 2010;10:842-857.
43. Barbacid M. ras genes. *Annu Rev Biochem*. 1987;56:779-827.
44. Santos E, Nebreda AR. Structural and functional properties of ras proteins. *FASEB J*. 1989;3:2151-2163.
45. Lassar AB, Thayer MJ, Overell RW, Weintraub H. Transformation by activated ras or fos prevents myogenesis by inhibiting expression of MyoD1. *Cell*. 1989;58:659-667.
46. Klesse LJ, Parada LF. p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. *J Neurosci*. 1998;18:10420-10428.
47. Terada K, Kaziro Y, Satoh T. Ras is not required for the interleukin 3-induced proliferation of a mouse pro-B cell line, BaF3. *J Biol Chem*. 1995;270:27880-27886.
48. Johnson L, et al. K-ras is an essential gene in the mouse with partial functional overlap with N-ras [published erratum appears in *Genes Dev* 1997 Dec 1;11(23):3277]. *Genes Dev*. 1997;11:2468-2481.
49. Umanoff H, Edelmann W, Pellicer A, Kucherlapati R. The murine N-ras gene is not essential for growth and development. *Proc Natl Acad Sci U S A*. 1995;92:1709-1713.

50. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer*. 2007;7:295-308.
51. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997;88:593-602.
52. Cichowski K, Jacks T. NF1 tumor suppressor gene function: narrowing the GAP. *Cell*. 2001;104:593-604.
53. Kolch W, Heidecker G, Lloyd P, Rapp UR. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. *Nature*. 1991;349:426-428.
54. Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol*. 2004;5:875-885.
55. Maurer G, Tarkowski B, Baccarini M. Raf kinases in cancer roles and therapeutic opportunities. *Oncogene*. 2011;30:3477-3488.
56. Dhillon AS, Hagan S, Rath O, Kolch W. MAP kinase signalling pathways in cancer. *Oncogene*. 2007;26:3279-3290.
57. Anjum R, Blenis J. The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol*. 2008;9:747-758.
58. Bermudez O, Pages G, Gimond C. The dual-specificity MAP kinase phosphatases: critical roles in development and cancer. *Am J Physiol Cell Physiol*. 2010;299:C189-C202.
59. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer*. 2011;11:558-572.
60. Beroukhim R, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
61. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.
62. Palanisamy N, Ateeq B, Kalyana-Sundaram S, et al. Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. *Nat Med*. 2010;16:793-798.
63. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 2009;9:537-549.
64. Van Beveren C, van Straaten F, Curran T, Muller R, Verma IM. Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular fos gene products have different carboxy termini. *Cell*. 1983;32:1241-1255.
65. Maki Y, Bos TJ, Davis C, Starbuck M, Vogt PK. Avian sarcoma virus 17 carries the jun oncogene. *Proc Natl Acad Sci U S A*. 1987;84:2848-2852.
66. Miller AD, Curran T, Verma IM. c-fos protein can induce cellular transformation: a novel mechanism of activation of a cellular oncogene. *Cell*. 1984;36:51-60.
67. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 2002;4:E131-E136.
68. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6:635-645.
69. Dang CV. MYC on the path to cancer. *Cell*. 2012;149:22-35.
70. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. *Semin Cancer Biol*. 2006;16:318-330.
71. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Mol Cell*. 2010;37:299-310.
72. Strasser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J*. 2011;30:3667-3683.
73. Cantley LC. The phosphoinositide 3-kinase pathway. *Science*. 2002;296:1655-1657.
74. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
75. Chang HW, et al. Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science*. 1997;276:1848-1850.
76. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol*. 2012;13:283-296.
77. Taniguchi T. Cytokine signaling through nonreceptor protein tyrosine kinases. *Science*. 1995;268:251-255.
78. Wang X, Lupardus P, Laporte SL, Garcia KC. Structural biology of shared cytokine receptors. *Annu Rev Immunol*. 2009;27:29-60.
79. Stark GR, Darnell Jr JE. The JAK-STAT pathway at twenty. *Immunity*. 2012;36:503-514.
80. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene*. 2002;285:1-24.
81. Quintas-Cardama A, Kantarjian H, Cortes J, Verstovsek S. Janus kinase inhibitors for the treatment of myeloproliferative neoplasias and beyond. *Nat Rev Drug Discov*. 2011;10:127-140.
82. Cocault L, Bouscary D, Le Bousse Kerdiles C, et al. Ectopic expression of murine TPO receptor (c-mpl) in mice is pathogenic and induces erythroblastic proliferation. *Blood*. 1996;88:1656-1665.
83. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med*. 2006;3, e270.
84. Yan R, Luo H, Darnell Jr JE, Dearolf CRA. JAK-STAT pathway regulates wing vein formation in Drosophila. *Proc Natl Acad Sci U S A*. 1996;93:5842-5847.
85. Lacronique V, Boureux A, Valle VD, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*. 1997;278:1309-1312.
86. Pilati C, Amessou M, Bihl MP, et al. Somatic mutations activating STAT3 in human inflammatory hepatocellular adenomas. *J Exp Med*. 2011;208:1359-1366.
87. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer*. 2009;9:798-809.
88. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. *Nat Rev Cancer*. 2007;7:79-94.
89. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L. GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature*. 1989;340:692-696.
90. Lyons J, Landis CA, Harsh G, et al. Two G protein oncogenes in human endocrine tumors. *Science*. 1990;249:655-659.
91. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. *Cancer Res*. 1999;59:1152-1159.
92. Uemura H, Hasumi H, Ishiguro H, Teranishi J, Miyoshi Y, Kubota Y. Renin-angiotensin system is an important factor in hormone refractory prostate cancer. *Prostate*. 2006;66:822-830.
93. Clerc P, Leung-Theung-Long S, Wang TC, et al. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia. *Gastroenterology*. 2002;122:428-437.
94. Lappano R, Maggiolini M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov*. 2011;10:47-60.
95. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev*. 2003;24:389-427.
96. van Essen M, Krenning EP, Kam BL, de Jong M, Valkema R, Kwekkeboom DJ. Peptide-receptor radionuclide therapy for endocrine tumors. *Nat Rev Endocrinol*. 2009;5:382-393.
97. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127:469-480.
98. Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev*. 2008;22:2308-2341.
99. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature*. 2005;434:843-850.
100. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
101. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell*. 2009;17:9-26.
102. Nusse R, Varmus HE. Wnt genes. *Cell*. 1992;69:1073-1087.
103. Brown AM, Wildin RS, Prendergast TJ, Varmus HE. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. *Cell*. 1986;46:1001-1009.
104. Bafico A, Gazit A, Wu-Morgan SS, Yaniv A, Aaronson SA. Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. *Oncogene*. 1998;16:2819-2825.
105. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA. An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell*. 2004;6:497-506.
106. Akiri G, Cherian MM, Vijayakumar S, Liu G, Bafico A, Aaronson SA. Wnt pathway aberrations including autocrine Wnt activation occur at high frequency in human non-small-cell lung carcinoma. *Oncogene*. 2009;28:2163-2172.

107. Vijayakumar S, Liu G, Rus IA, et al. High-frequency canonical Wnt activation in multiple sarcoma subtypes drives proliferation through a TCF/beta-catenin target gene, CDC25A. *Cancer Cell*. 2011;19:601-612.
108. Polakis P. Drugging Wnt signalling in cancer. *EMBO J*. 2012;31:2737-2746.
109. Seshagiri S, Stawiski EW, Durinck S, et al. Recurrent R-spondin fusions in colon cancer. *Nature*. 2012;488:660-664.
110. Lum L, Beachy PA. The Hedgehog response network: sensors, switches, and routers. *Science*. 2004;304:1755175-1755179.
111. Briscoe J, Therond P. Hedgehog signaling: from the *Drosophila* cuticle to anticancer drugs. *Dev Cell*. 2005;8:143-151.
112. Ingham PW, Nakano Y, Seger C. Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat Rev Genet*. 2011;12:393-406.
113. Ryan KE, Chiang C. Hedgehog secretion and signal transduction in vertebrates. *J Biol Chem*. 2012;287:17905-17913.
114. Hahn H, et al. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell*. 1996;85:841-851.
115. Xie J, Murone M, Luoh SM, et al. Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature*. 1998;391:90-92.
116. Yauch RL, Gould SE, Scales SJ, et al. A paracrine requirement for hedgehog signalling in cancer. *Nature*. 2008;455:406-410.
117. Ng JM, Curran T. The Hedgehog's tale: developing strategies for targeting cancer. *Nat Rev Cancer*. 2011;11:493-501.
118. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heal of cancer. *Science*. 2002;297:63-64.
119. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467:596-599.
120. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
121. Poulikakos PI, Rosen N. Mutant BRAF melanomas—dependence and resistance. *Cancer Cell*. 2011;19:11-15.
122. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012;483:100-103.

Nelson E. Brown and Philip W. Hinds

3

Tumor Suppressor Genes

Introduction

Over the past 40 years, efforts to understand the underlying rules that govern the transformation of somatic cells into their malignant counterparts have led to the identification of discrete alterations in genes and gene products that, in combination, are responsible for the characteristic hallmarks of cancer.[1] Broadly speaking, cancer-associated genes can be classified into three groups: oncogenes, tumor suppressor genes, and genes responsible for maintaining genome stability. Oncogenes encode the constitutively active or overexpressed versions of otherwise normal cellular proteins involved in cell growth and proliferation (i.e., tyrosine kinase receptors, transduction kinases, and small GTPases). The "gain of function" capabilities of oncogenes are acquired as a result of genetic and epigenetic mechanisms, including chromosomal translocations, gene amplifications, missense activating mutations, and demethylation of gene promoters. Regardless of the activating mechanism, an oncogene always behaves as a dominant allele (a single allele suffices) in its ability to confer malignant properties on cells.[2]

In contrast to oncogenes, tumor suppressor genes encode proteins that are functionally integrated into pathways that prevent unscheduled cell proliferation, stimulate cell death, or trigger the induction of permanent cell cycle arrest.[2] As expected, tumor suppressor genes may act as negative regulators of oncogenes. In many cases, they are responsible for the orchestration of cell cycle checkpoints that ensure faithful cell division under normal or stress-induced conditions.[3] The involvement of tumor suppressor genes in the tumorigenic process is only apparent following complete or partial loss of gene function, which commonly requires the inactivation of both parental alleles in a single cell. This recessive behavior explains the fact that the mutant alleles of these genes can be passed through the germline and cause inherited forms of cancer predisposition in humans.[2,4] Inactivating mechanisms of tumor suppressor genes include deletions, nonsense, and missense mutations and methylation-mediated gene silencing.

The third class of cancer-associated genes comprises those primarily involved in cellular processes that maintain basal levels of genomic or chromosomal stability. The proficiency of a cell in accurately repairing various forms of genomic insult depends on its ability to sense acute genomic damage, usually in the form of single- or double-strand DNA breaks, and to mobilize specific repair enzymatic complexes to sites of DNA damage.[5] As expected, the inactivation of gene products involved in these processes leads to an increase in the rates of spontaneous mutations. This "mutator phenotype" can, in turn, contribute to the accumulation of mutations in oncogenes and tumor suppressor genes.[2] Similar to tumor suppressor genes, defects in genes involved in genomic surveillance underlie a variety of cancer-prone genetic disorders, most of them inherited in a recessive fashion. For example, nucleotide excision repair (a process responsible for the repair of single-strand breaks and crosslinks in DNA produced by UV radiation or chemical mutagens) is defective in *xeroderma pigmentosum*, a group of human disorders associated with the development of tumors on sun-exposed skin.[6]

A major contributor to genomic instability in human cancers is the inactivation of *TP53*, the gene encoding the p53 transcription factor.[7] As mentioned later in this chapter, p53 is normally induced in response to a variety of stresses, including DNA damage. Depending on the cell type and the magnitude of the damage, p53 activation can result in cell cycle arrest, senescence, or cell death (apoptosis), processes that effectively prevent the propagation of damaged DNA within a cell population or give an individual cell time to repair the damage. Hence, cells lacking p53 may continue to replicate damaged DNA, increasing the chances of accumulating potentially oncogenic mutations in other loci.[2,4,7] Notwithstanding its crucial role in sensing and responding to genotoxic insults, for historical reasons p53 is often described as a prototypical tumor suppressor gene.

Tumor Suppressor Genes: A Historical Perspective

From a historical point of view, the articulation of the modern concept of tumor suppressor gene (TSG) was possible through the convergence of three major lines of research: somatic cell hybridization experiments, the detection of loss of heterozygosity (LOH) in tumors, and the study of highly penetrant familial cancers.[8,9] It is worth mentioning, however, that the existence of TSGs had already been anticipated early in the 20th century by Theodor Boveri (1862-1915), one of the founders of the chromosomal theory of inheritance (the modern concept of the gene had not been developed at the time). Boveri suggested that the uncontrolled growth characteristic of tumors arises as a result of an incorrect chromosomal dosage, which could be explained by an abnormal segregation of chromosomes during cell division. Thus, "growth inhibitory chromosomes" are removed from cells during the process of tumorigenesis. As a corollary, Boveri suggested that these inhibitory chromosomes were part of a mechanism that kept normal cells in a proliferation-arrested state unless they were stimulated to divide.[10] It was not until the mid-20th century, however, that more sensitive cytogenetic techniques allowed the identification of LOH (loss of heterozygosity), indicative of chromosomal loss, in human tumors.

The next piece of evidence linking loss of function mutations and tumorigenesis came from somatic cell hybridization experiments performed in the early 1970s.[11-13] The crucial observation here was that cell hybrids generated through fusion of normal somatic cells with tumor-derived cells were usually nontumorigenic. The dominant effect of normal traits over the malignant phenotype seemed to indicate that loss of growth-regulatory genetic information had contributed to the transformation of the parental tumor cell line in the first place. Conversely, the neoplastic phenotype could be reversed following the reacquisition of the normal complement of genetic information.

Ultimately, however, it was the study of familial cases of cancer predisposition that led to the identification, and subsequent cloning and characterization, of the first TSGs. Highly penetrant cancer susceptibility syndromes constitute a small group of inherited disorders in which the affected individuals develop a unique type of tumor (or a narrow set of tumors) with an unusual high incidence and at a younger age compared with sporadic (noninherited) cases.[14] Although cancer-associated syndromes display a dominant mode of inheritance, the experimental evidence gathered from in vitro cell fusion experiments and LOH analyses seemed to indicate that TSGs acted in a recessive manner at the cellular level. A theoretical explanation for this paradox was first provided by Alfred Knudson and became later known as the "two-hit" hypothesis.[8,15]

In 1971, Knudson was studying the epidemiology of retinoblastoma, a relatively rare pediatric tumor that originates in the fetal retina. Retinoblastoma is associated with an inherited predisposition in approximately 40% of the cases. Most children with an affected parent develop multiple retinoblastomas in both eyes, which are diagnosed at a younger age compared with the sporadic forms of the disease. In contrast, children diagnosed with sporadic retinoblastoma (children with no family history of the disease, about 60% of the cases) show unilateral involvement and typically a single tumor in the affected eye. Based on these differences, Knudson postulated that the cell of origin of retinoblastoma (the retinoblast) must undergo two critical genetic events at a single locus in order to initiate a tumor. Because the first mutation or "hit" is already present in every somatic cell (including retinoblasts) of an individual with hereditary retinoblastoma, an inactivating mutation of the remaining allele (the second "hit") would be sufficient to drive tumor formation. The increased probability of tumor initiation in the context of a large population of already mutated retinoblasts helps explain the multiplicity of tumors per retina and the characteristic early onset of hereditary retinoblastoma. In cases of sporadic retinoblastoma, on the other hand, the hypothesis postulated that both hits must take place in a single somatic cell, itself a much less probable event, thus explaining the unilateral involvement and the lower number of tumors per retina.[15]

Evidence for the "two-hit" model was later provided by cytogenetic analyses of blood samples from patients with inherited retinoblastoma. These studies identified germline deletions of chromosomal band 13q14 in these patients, and similar changes were subsequently found in sporadic tumors, providing strong support for the notion that the same genetic locus was affected in both variants of the disease. Although several mechanisms were initially proposed to explain the second hit, their final documentation was only possible through DNA restriction fragment length polymorphism (RFLP) analysis. RFLP-based studies demonstrated that LOH achieved through deletion, mutation, or recombination can all occur as second events in retinoblastomas.[16,17] Finally, these studies provided the necessary molecular clues that led to the cloning of the retinoblastoma susceptibility gene (*RB-1*) in 1986.[18]

The protein encoded by *RB-1* (pRB) is now recognized as a key suppressor of cell cycle progression.[19] Evidence for pRB's function dates back to studies on cellular immortalization mediated by viral oncoproteins, including the simian virus 40 (SV40) large T antigen. These studies demonstrated that cellular immortalization was in part a result of the direct inactivation of pRB by the SV40 large T antigen. Because immortalized cells were especially sensitive to transformation by several oncogenes, the natural conclusion was that

inactivation of pRB might represent a common requirement for the initiation of most cancers. However, it soon became evident that inactivating mutations in *RB-1* are found in a rather narrow group of human malignancies, most typically in small-cell lung carcinomas and osteosarcomas. As mentioned later in this chapter, we know now that deregulation of numerous cancer-associated genes can lead to the inactivation of pRB in more indirect ways, a hallmark shared by virtually all cancers.[19,20]

At around the same time, the status of *TP53* (also known as *p53*) as a TSG was also established. Its gene product, the transcription factor p53, was originally identified as an interacting partner of the SV40 large T antigen. Although the mechanisms of tumorigenesis involving p53-large T antigen interaction initially suggested that *TP53* acted as an oncogene, further experimental evidence challenged this notion. For example, wild-type p53 was shown to act as a suppressor of transformation in cultured cells, and genetic rearrangements at the *TP53* locus, which resulted in loss of function instead of activation, were discovered in some leukemia cell lines.[21] In addition, it was suggested that the interaction between p53 and the SV40 large T antigen resulted in loss of p53 function, in a manner reminiscent of pRB inactivation. Nevertheless, it was not until *TP53* was mapped within a region consistently deleted in human tumors that the gene gained its final recognition as a bona fide TSG. Seminal among these studies was the discovery that *TP53* was biallelically deleted in human colorectal cancers, which was soon followed by the identification of mutations in other malignancies.[2] Loss-of-function mutations in *TP53* were then linked to Li-Fraumeni syndrome, a known dominantly inherited condition that predisposes individuals to several cancers, most typically breast cancer.[22] Over the following 40 years, an explosion of research has confirmed *TP53* as one of the most frequently mutated genes in human tumors. As mentioned later, p53 is involved in the orchestration of a variety of tumor-suppressive processes, including cell cycle arrest, apoptosis, and senescence. Deregulation of each one of these processes following p53 inactivation can thus increase the probability that a cell will become malignant.[4]

In summary, the *RB-1* and *p53* paradigms defined three important properties of "classic" TSGs: (1) TSGs are recessive at the cellular level, with biallelic inactivation typically found in tumors; (2) inheritance of a single mutant allele increases tumor susceptibility because only one additional inactivating event is required for complete loss of gene function; and (3) the same gene is frequently inactivated in sporadic cancers. Theoretically, reversion of the tumorigenic phenotype following the reintroduction of the relevant TSG into a cancer cell may also serve as a functional criterion to classify a gene as tumor suppressor. However, this requirement is not always met experimentally, presumably because loss of a TSG can allow further genetic changes that may confer resistance to its restoration at a later time.[4]

These principles served as guidance for the identification and cloning of other cancer susceptibility loci. Table 3-1 lists selected TSGs along with the function of their encoded proteins, the cancer syndrome they are associated with, and examples of sporadic cancers associated with their loss of function.

Complications of Tumor Suppression

Moderate and Low Penetrance Cancer Susceptibility Loci

Despite their importance in the discovery of TSGs, highly penetrant cancer syndromes account for a relatively small proportion of human malignancies (typically less than 0.1%). In fact, the excess in familial risk for some types of cancer has remained, to a large extent, unexplained. For example, breast cancer shows a pattern in which relative risk increases by two- to threefold in first-degree relatives of early-onset cancer cases. However, mutations in *BRCA1* and *BRCA2* account for only ~20% of this overall risk increase. It has been proposed that much of the inherited risk may result from a polygenic mode of cancer predisposition. In this scenario, multiple loci, each one having a modest individual effect, may ultimately dictate the relative risk of an individual to develop cancer.[9] In support for this idea, recent reports have identified susceptibility alleles associated with a wide range of risk in human populations. For example, the screening of genes associated with *BRCA1*- and *BRCA2*-dependent pathways demonstrated "moderate" breast cancer risk in individuals who are heterozygous for allelic variants of *CHEK2*. These rare alleles function dominantly, each conferring a moderate but detectable increase in the relative risk of developing breast cancer.[23,24] More recently, genome-wide association (GWA) studies based on differences in single-nucleotide polymorphism (SNP) across human populations have begun to identify "low-penetrance" cancer susceptibility loci for the most common types of cancer.[25,26]

Haploinsufficiency

The two-hit hypothesis has been challenged by recent studies indicating that many chromosomal deletions in cancer cells consistently affect a single allele. Although these monoallelic deletions were initially considered to be mere "passenger events" with no actual causal role, numerous genes that behave as TSGs in vitro have been identified in these regions. This observation implies that a single-copy mutation or loss of these loci might be sufficient to explain their

Table 3-1 Representative Tumor Suppressor Genes

Gene	Familial Cancer Syndrome	Protein Function	Sporadic Tumors with Mutations
RB1	Hereditary retinoblastoma	Transcriptional co-repression	Sporadic retinoblastoma, osteosarcoma, small-cell lung carcinoma (SCLC), breast carcinoma, bladder carcinoma
TP53 (p53)	Li-Fraumeni syndrome	Transcription factor	>50% of all cancers
APC	Familial adenomatous polyposis (FAP)	Wnt signaling, degrades beta-catenin	Colorectal cancer, gastric cancer
WT1	Wilms tumor (nephroblastoma)	Transcription factor	Pediatric kidney cancer
NF1	Neurofibromatosis type 1	GTPase activating protein for Ras	Sarcoma, gliomas
NF2	Neurofibromatosis type 2	Membrane-cytoskeleton binding protein	Schwannoma, meningioma, ependymoma
INK4a (p16)	Familial melanoma	CDK4/6 inhibitor (pRB activation)	Many
ARF	Melanoma	MDM2 antagonist (p53 stability)	Many
VHL	Von Hippel-Lindau syndrome (renal tumor)	E3 ligase recognition factor for HIF1 (hypoxia response)	Renal carcinoma (clear cell), cerebellar hemangio-sarcoma, pheochromocytoma
PTEN	Cowden disease	Lipid phosphatase (phosphoinositide metabolism)	Glioblastoma, endometrial carcinoma, prostate carcinoma, breast carcinoma, thyroid cancer
LKB1 (STK11)	Peutz-Jeghers syndrome	Energy (glucose) sensor kinase (phos-phorylates AMPK)	Non–small-cell lung carcinoma (NSCLC) cervical carcinoma
TSC1, TSC2	Tuberous sclerosis (hamartomas)	GTPase activating complex for Rheb (mTORC1 inhibition)	Renal cell carcinoma (rare), angiofibroma
BRCA1	Familial breast and ovarian cancer	DNA repair, cell cycle control (genomic stability)	Unknown
BRCA2	Familial breast and ovarian cancer	DNA repair (homologous recombination, genomic stability)	Unknown
PTCH	Nevoid basal cell carcinoma (Gorlin) syndrome	Hedgehog signaling (transmembrane receptor)	Basal cell (skin) carcinoma, medulloblastoma, rhabdomyosarcoma
SMAD4 (DPC4)	Familial juvenile polyposis (hamartomas)	TGF-beta signaling (transcription factor)	Pancreatic and colon carcinomas
MSH2, MLH1, PMS1, PMS2	Hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome)	DNA mismatch repair	Endometrial, ovarian, gastric, hepatobiliary, and urinary tract cancer
CDH1 (E-cadherin)	Familial gastric carcinoma (diffuse type)	Cell-cell adhesion	Gastric cancer, lobular breast cancer

tumorigenic effect, a genetic property known as *haploinsufficiency*. For some alleles, haploinsufficiency may even confer a relative advantage to cells, most typically in situations where complete loss of function leads to apoptosis or senescence. For example, whereas a monoallelic deletion of the tumor suppressor *PTEN* is sufficient to produce prostate cancer in mice, loss of both parental alleles triggers a p53-dependent senescence program.[8,27] Alternatively, a monoallelic mutation may confer *dominant-negative* capabilities on TSG products. In this modality, the mutant protein may negatively interfere with the function of the wild-type protein produced by the unaffected allele. Because dominant-negative mutations can result in considerable loss of function, there is no selective pressure in tumors to inactivate or delete the wild-type allele. A classic example of a dominant-negative effect is provided by mutant p53. As part of its function as a transcription factor, wild-type p53 binds DNA as a tetramer, a capacity impaired in mutated p53 because of missense mutations affecting the DNA binding domain. Although the wild-type and the mutant p53 proteins are still able to form hetero-oligomers in cells harboring monoallelic mutations, these complexes show impaired DNA association and transcriptional activity, resulting in loss of p53 function.[22]

A third interpretation of the role of haploinsufficiency in tumorigenesis is based on the exquisite sensitivity of cells to even small changes in the levels of some cancer-associated proteins. Thus, a 50% functional reduction in a TSG product may be sufficient to endow a cell with a relative advantage for proliferation. Experimental evidence for this dosage-sensitivity effect has been shown for several TSGs, including *TP53*, *BRCA1*, *BRCA2*, and *PTEN*. For example, a subset of tumors arising in $p53^{+/-}$ mice, or in patients with

Li-Fraumeni syndrome, retain the wild-type allele, suggesting that haploinsufficiency of *TP53* may be sufficient for tumor initiation.[28-30] Finally, the pro-tumorigenic effect imparted by haploinsufficiency might also be dependent on the loss or gain of function of other alleles. An example of this interaction is illustrated in mouse models of *Pten* and *Tp53* deficiency. Haploinsufficiency of *Pten* in the context of wild-type p53 enhances proliferation and subsequent transformation of prostate epithelial cells. In contrast, complete loss of *Pten* in this tissue triggers a p53-dependent senescence program, with tumors arising only after *Tp53* inactivation.[8]

Interconnecting the pRB, p53, and mTORC1 Pathways

Similar to an evolutionary process driven by natural selection, the acquisition of the malignant phenotype can be best described as an iterative process of somatic mutation followed by clonal expansion. The current consensus is that normal cells must acquire at least four distinct mutational or epigenetic events (including gain- and loss-of-function alterations) in order to bypass proliferative control. These events alter critical signaling networks that ultimately control the decision of a cell to proliferate, senesce, or die. The discovery of these pathways represented a major success in the history of cancer biology, for it made possible the assignment of a growing number of cancer-associated gene products to a much more limited number of interacting networks.[2] Because of their high association with human malignancies, a more detailed description of pathways centered on pRB, p53, and mTORC1 is used in the next sections to illustrate the emergent complexities of tumor suppression. Whenever necessary, the relevant nodes interconnecting these pathways are stressed.

The pRB Tumor Suppressor Pathway

Despite the low incidence of *RB-1* mutations, it has become clear that deregulation of the pRB pathway is present in most (if not all) human cancers. Deregulation can occur through gain or loss of function of various components of the pathway. In most cases, these alterations ultimately result in pRB phosphorylation, a modification necessary for G1-S cell cycle transition.[31,32] In G1, *hypo*phosphorylated (active) pRB forms repressive complexes with E2F transcription factors at gene promoters of S-phase genes, a function in part mediated by the recruitment of histone-modifying complexes to these sites. These modifications result in a silent chromatin configuration that effectively turns off S-phase genes. Signals that promote proliferation must reverse this inhibition, and they do so through pRB phosphorylation.[19] In mammalian cells, pRB is phosphorylated by cyclin-dependent kinases (CDKs), a group of enzymes that require the binding of short-lived proteins called *cyclins* in order to become active. As shown in Figure 3-1, CDK4 and CDK6 form active complexes with D-type cyclins in *early* G1, whereas CDK2 is activated by E-type cyclins in *late* G1 and S phase. Following phosphorylation, *hyper*phosphorylated (inactive) pRB releases E2F transcription factors, which leads to derepression of S-phase genes (see Figure 3-1). Predictably, cyclins and CDKs act as oncoproteins in human cancers. For example, *CCD1* (encoding cyclin D1) is amplified or overexpressed in more than 50% of human breast cancers.[31,33]

CDKs are also subject to negative regulation by two families of CDK inhibitors. Members of the INK4 family, such as $p16^{INK4a}$, bind to CDK4 or CDK6 and prevent their association with D-type cyclins. On the other hand, members of the CIP1/KIP1 family, which include $p21^{CIP1}$ and $p27^{KIP1}$, form inhibitory complexes with CDK2 and cyclin E.[31] In both cases, CDK inhibition results in pRB activation and cell cycle arrest (see Figure 3-1). As expected, many CDK inhibitors behave as tumor suppressors in human malignancies. For example, *CDKN2A*, the gene encoding $p16^{INK4a}$, is frequently deleted or epigenetically silenced in cases of familial melanoma and in several sporadic tumors.[34]

The *CDKN2A*-containing locus is also remarkable in that it encodes a second, structurally and functionally unrelated protein from an alternative reading frame and a different gene promoter.[35] This alternative reading frame protein (ARF, also called $p14^{ARF}$ in humans and $p19^{Arf}$ in mice) stabilizes p53 by directly inactivating MDM2, the E3-ubiquitin ligase that targets p53 for degradation. Therefore, loss-of-function mutations affecting *ARF*, as well as gain-of-function mutations in *MDM2*, can both have a similar destabilizing effect on p53. *ARF*-specific inactivating mutations have been described in a subset of human melanomas; *MDM2* amplifications, on the other hand, are common in human sarcomas.[2] Notice that the unique genomic arrangement of the *CDKN2A* locus, encoding both $p16^{INK4a}$ and $p14^{ARF}$, means that deletions of this locus would simultaneously compromise the functions of the pRB and p53 pathways. Hence, in a manner reminiscent of the inactivation of pRB and p53 by the SV40 large T antigen, cells harboring certain *CDKN2A* mutations display an enhanced proliferative capacity in vitro, which in many cases is sufficient for the establishment of continuously proliferating (immortal) cell lines. This immortalization step, in turn, sensitizes cells to oncogene-mediated transformation.[36]

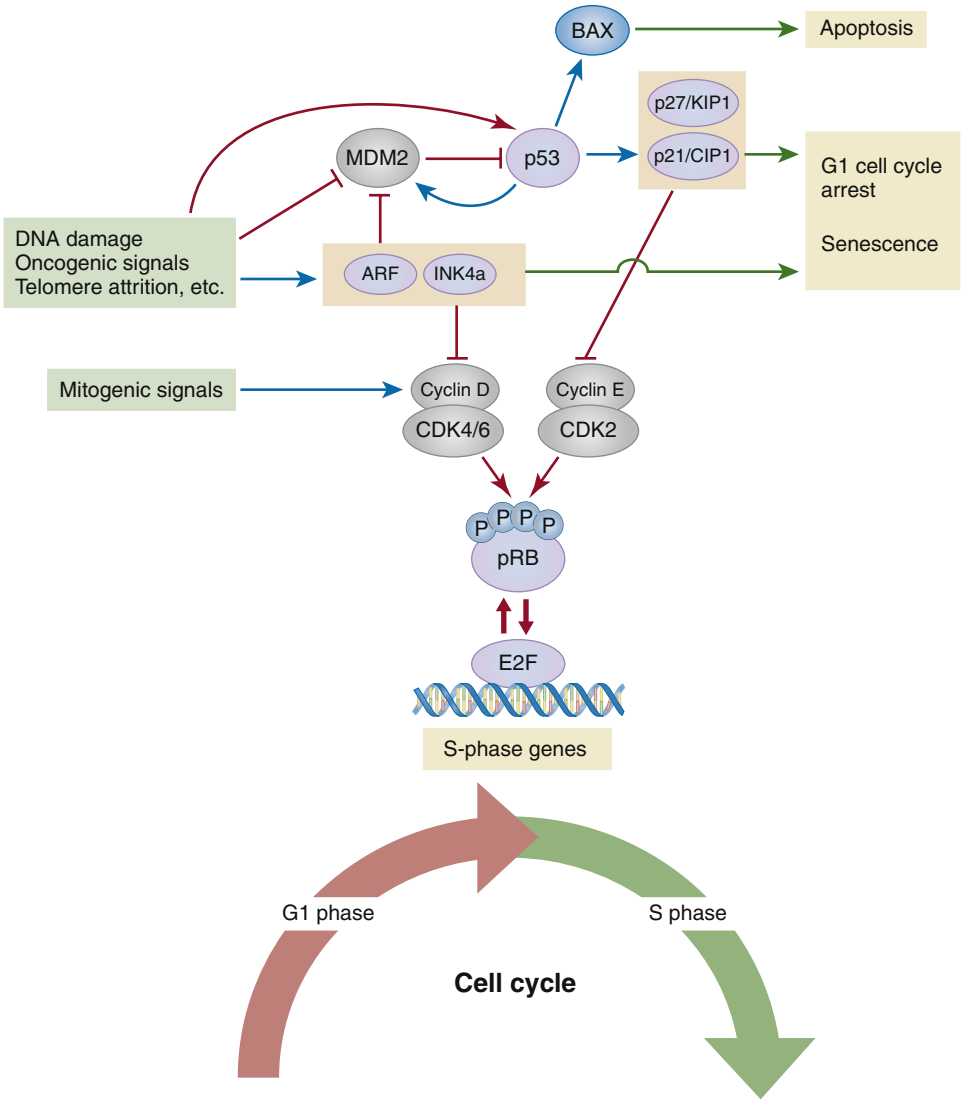


Figure 3-1 The pRB and p53 tumor suppressor pathways Under physiological conditions, signals that promote proliferation (i.e., growth factors) induce the expression of D-type cyclins, short-lived proteins required for the activation of CDK4 and CDK6 (CDK4/6) in early G1. In cooperation with cyclin E–CDK2 complexes, cyclin D1–CDK4/6 complexes contribute to the phosphorylation-mediated inactivation of pRB and the derepression of E2F-responsive (S-phase) genes. A variety of stresses, including DNA damage, telomere erosion, and oncogenic stress, turn on signaling cascades that activate the *CDKN2A* locus. ARF, one of the tumor suppressors produced from this locus, inhibits MDM2 (the E3-ubiquitin ligase that targets p53 for degradation), resulting in the stabilization of p53 and the induction of p53-dependent transcriptional programs (the transcriptional activity of p53 can also be affected by numerous posttranslational modifications that, for simplicity, are not depicted here). Depending on the cell type and/or the nature and magnitude of the stress, p53 can induce the expression of genes that promote apoptosis or, alternatively, genes involved in cell cycle arrest, such as the gene encoding the CDK inhibitor $p21^{CIP1}$. $p21^{CIP1}$, in turn, inhibits cyclin E–CDK2 complexes and leads to pRB-dependent cell cycle arrest. The second tumor suppressor encoded by the *CDKN2A* locus, $p16^{INK4a}$ (INK4a), is also induced in response to stress (with or without concomitant induction of ARF), most typically oncogenic-dependent stress. By antagonizing CDK4/6, $p16^{INK4a}$ also activates pRB and prevents entry into S phase. *Red* arrows and *red* T-shaped connectors indicate activating and inhibitory processes, respectively; *blue* arrows indicate transcriptional induction; *green* arrows point to final cellular outcomes.

The p53 Tumor Suppressor Pathway

As already mentioned, p53 is a multifunctional transcription factor that is activated in response to a variety of stress conditions, including DNA damage, activated oncogenes, telomere shortening, spindle damage, hypoxia, and metabolic stress.[7] Depending on the cell type and the magnitude or nature of the stress, the transcriptional program activated by p53 can lead to one of three outcomes: cellular senescence, G1/G2 arrest, or apoptosis (see Figure 3-1). Mirroring the diversity of stress signals is the equally diverse number of intracellular networks involved in the transmission of these signals to p53. What all these networks have in common, however, is the ability to activate p53 via posttranslational modifications (including phosphorylation, acetylation, methylation, and sumoylation) that enhance p53's capacity to transcribe target genes and also increase the half-life of the protein. We have seen already that one way to stabilize p53 involves the ARF-dependent inhibition of MDM2.

Among numerous p53 targets, the gene encoding $p21^{CIP1}$ (*CDKN1A*) is probably the best-known example that connects p53 to the pRB pathway (p53-mediated upregulation of $p21^{CIP1}$ leads to CDK inhibition and pRB-mediated cell cycle arrest). In addition to targets involved in cell cycle arrest, p53 also induces genes that control or promote apoptosis (such as *BAX*, *PUMA*, and *PIG3*), a cellular outcome favored by some cell types undergoing extensive DNA damage.[37]

Given the pleiotropic roles of p53 in tumor suppression, it is not surprising that mutations in *TP53* are almost a universal feature of human cancers. Most of these mutations (~74%) are missense mutations that fall within the DNA-binding domain and therefore disrupt p53's ability to bind cognate promoter sequences on target genes.[22]

pRB and p53 Pathways in Action: Cellular Senescence

The connection between the two major tumor suppressors, pRB and p53, and cellular senescence is a recurrent theme throughout this chapter. Because of the growing awareness that cellular senescence constitutes a physiological barrier against tumor initiation and progression, a brief description of two related variants of senescence, and their connection with the pRB and p53 pathways, is necessary (see Figure 3-1).

Cellular senescence is a form of *irreversible* cell cycle arrest associated with a unique gene expression profile and distinctive cell morphology.[38] The first description of this phenomenon can be traced back to efforts to propagate human cells in vitro. Explanted human cells typically proliferate for a variable period of time but eventually undergo "replicative" senescence in response to the attrition of telomeres (the protective chromosomal termini) that accompanies each cell division. Telomere disruption triggers a stress response that in many respects is identical to a p53-dependent DNA damage response (DDR).[39] Before the signs of DDR are evident, however, an early induction of $p16^{INK4a}$ also contributes to limit the proliferative capacity of human cells, which is illustrated by the significant delay in the onset of replicative senescence observed in $p16^{INK4a}$-deficient cells. Nonetheless, in order to completely bypass senescence, human cells must also overcome the p53 pathway and reactivate telomerase (the enzyme responsible for telomere maintenance).[38,40]

A form of replicative senescence associated with the induction of $p16^{INK4a}$ and p53 is also observed in primary mouse cells, but, in contrast to human cells, the inactivation of just one of these tumor suppressors suffices to bypass senescence. Because murine cells constitutively express telomerase, senescence in this case might be a response to nonphysiological culture conditions (i.e., high oxygen levels).[38]

In addition to replicative senescence, primary cells also undergo "premature" senescence in response to oncogenic stress (i.e., overexpression of activated *HRAS*), a phenomenon known as "oncogene-induced senescence" (OIS). Similar to replicative senescence, OIS is also dependent on the induction of $p16^{INK4a}$ and p53, although the relative contribution of these tumor suppressors varies between species and cell types.[38,41] Recently, OIS has been confirmed as a barrier to tumorigenesis in vivo. For example, benign melanocytic tumors called "nevi" are associated with activating mutations in the oncoprotein BRAF. These lesions are typically positive for markers of senescence, including elevated levels of $p16^{INK4a}$. As expected, loss of $p16^{INK4a}$ in these lesions accelerates the formation of malignant melanomas.[42,43]

The mTORC1-Dependent Pathways

Alterations in a cell's ability to respond to metabolic and growth-promoting signals constitute common features of cancers. At the center of these regulatory networks is mTORC1 (mammalian target of rapamycin complex 1), a kinase complex that integrates nutrient and growth factor availability with downstream effectors involved in cell growth and proliferation. mTORC1's main function is to promote biosynthetic processes (i.e., protein synthesis and ribosome biogenesis) that increase cell mass. Two pathways that converge on mTORC1 (the PI3K- and AMPK-dependent pathways) are particularly relevant to cancer[44,45] (Figure 3-2).

Activation of tyrosine kinase receptors, most classically insulin-like growth factor receptor (IGFR), activates the lipid kinase PI3K (class I phosphatidylinositol-3-kinase), an enzymatic complex that catalyzes the production of phosphatidylinositol-3-phosphate (PIP3). The local increase in PIP3 is in turn required for the activation of AKT kinases. AKTs inhibit the GTPase activating complex TSC (tuberous sclerosis complex, composed of TSC1 and TSC2 subunits), a process required to keep Rheb (a small GTPase) in its GTP-bound (active) conformation. As a result, AKT-mediated inactivation of TSC increases the pool of active Rheb, which in turn enables mTORC1 activation (see Figure 3-2). AKT-mediated phosphorylation of substrates other than TSC can also affect proliferation by controlling pRB activity. These substrates include the FoxO (Forkhead box O) family of transcription factors and GSK3-β (glycogen synthase kinase 3-beta). Under basal conditions, FoxOs induce the expression of the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, whereas GSK3-β participates in the degradation of cyclin D1 (see Figure 3-2). Therefore, AKT-mediated inactivation of FoxO and GSK3-β reduces the levels of CDK inhibitors

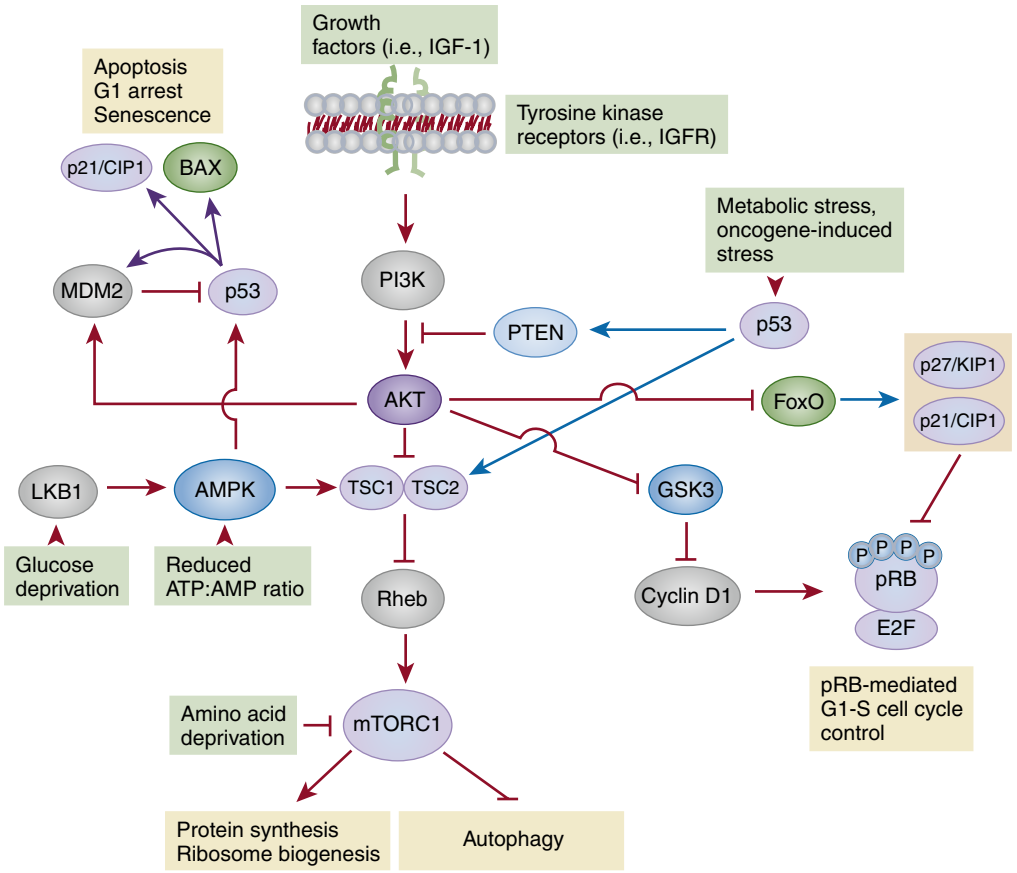


Figure 3-2 The PI3K-AKT-mTORC1 and LKB1-AMPK-mTORC1 pathways The main function of mTORC1 is to regulate biosynthetic pathways involved in cell growth, cell survival and proliferation. The proper function of mTORC1 depends on its ability to integrate inputs from two pathways: the PI3K-AKT axis, involved in the transduction of growth-promoting signals and the LKB1-AMPK axis, which monitors the energy status of the cell. Ligand-mediated activation of tyrosine kinase receptors (i.e., insulin-like growth factor receptor, IGFR) activates PI3K, which increases the local concentration of the lipid second messenger phosphatidylinositol-3-phosphate, PIP3. This activity is counteracted by PTEN, a lipid phosphatase that removes phosphate groups from PIP3. PI3K-mediated increase of PIP3 levels activates the AKT family of kinases, which inhibit the GTPase activating complex TSC (TSC1-TSC2). Inhibition of TSC allows Rheb to remain in its active (GTP-bound) conformation, resulting in mTORC1 activation. In addition, AKT-mediated phosphorylation of FoxO transcription factors and GSK3-β can also lead to pRB inactivation and cell cycle entry by reducing the expression of CDK inhibitors ($p21^{CIP1}$ and $p27^{KIP1}$) and increasing the stability of cyclin D1. The AMPK complex and its upstream activator LKB1 monitor the intracellular levels of glucose and ATP and modify the functional status of mTORC1 accordingly. In situations of metabolic stress, the resulting increase in the AMP:ATP ratio leads to the activation of LKB1, which phosphorylates and activates AMPK. Active AMPK can then inhibit mTORC1 through TSC-dependent and TSC-independent mechanisms. As shown here, in response to metabolic stress, p53 can directly induce the expression of TSC2 and PTEN, contributing to mTORC1 inhibition. Conversely, AMPK can also phosphorylate and activate p53. *Red* arrows and T-shaped connectors indicate activating and inhibitory processes, respectively; *blue* arrows indicate transcriptional induction.

and increases the levels of cyclin D1, outcomes that cooperate to promote cell cycle progression (see Figure 3-2).[46]

Normal cells must also implement mechanisms to reduce the PI3K-AKT activity in order to adjust the rates of protein synthesis to the available growth factors. One important mechanism of inhibition involves the activation of PTEN (phosphatase and tensin homologue), a lipid phosphatase that catalyzes the dephosphorylation of PIP3 and thus counteracts PI3K activation (see Figure 3-2).[44]

In cancer cells, activation of the PI3K-AKT-mTORC1 pathway can occur through several mechanisms. These include aberrant activation of tyrosine kinase receptors (TKRs), activating mutations in *PI3KCA* (the gene encoding the p110α subunit of PI3K), amplification of *AKT1*, downregulation of *TSC2*, or loss-of-function mutations in *PTEN*.[46] Loss of *PTEN* in particular constitutes a remarkably frequent alteration in human malignancies. Similar to other tumor suppressors, germline mutations in *PTEN* are also associated with inherited cancer syndromes (including Cowden disease), and hemiallelic loss of *Pten* in mice results in tumors arising in multiple epithelial tissues, including the intestine, prostate, and mammary gland. However, unlike other tumor suppressors, biallelic deletion of *Pten* can activate a p53-dependent senescence program that opposes transformation, suggesting that PTEN is an obligate haploinsufficient tumor suppressor. By comparison, mutations affecting other negative regulators of the PI3K-AKT-mTORC1 pathway are rare events in sporadic tumors, although germline deletions of *TSC1* or *TSC2* are known to be associated with cancer-prone syndromes.[44]

The second signaling pathway that modulates mTORC1 is the AMP-activated protein kinase (AMPK) pathway. During periods of *nutrient deprivation*, AMPK is activated by at least two mechanisms: (1) increased levels of AMP (due to a drop in ATP production) and (2) phosphorylation by LKB-1, a kinase that is itself activated in response to metabolic stress (i.e., glucose reduction) (see Figure 3-2). Active AMPK phosphorylates TSC2, leading to the activation of the TSC complex, inactivation of Rheb and mTORC1, and the consequent inhibition of protein synthesis and cell growth.[44] An important consequence of mTORC1 inhibition is a shift toward a predominantly catabolic metabolism. This is in part achieved through the induction of autophagy, the process in which organelles and protein complexes are targeted to the lysosome for degradation.[47]

It follows from this account that cancer cells must overcome the AMPK-dependent checkpoint in order to sustain proliferation under suboptimal metabolic conditions. Among the mechanisms of AMPK inactivation, loss of function of LKB-1 is probably the best known. Inactivating mutations of the gene encoding LKB1 (*STK11*) have been identified in patients with Peutz-Jeghers syndrome, a condition that predisposes individuals to the development of several types of cancer. Recently, *STK11* was also found mutated in sporadic cases of non–small-cell lung and cervical carcinomas.[44]

In recent years, it has become evident that most alterations in cancer-associated genes can result in metabolic changes that involve mTORC1-dependent pathways to variable degrees. In particular, the role of p53 in mediating adaptation to metabolic stress is becoming increasingly evident. Thus, inhibition of the AKT-mTORC1 axis, as well as activation of AMPK, can both lead to the induction of p53. The first mechanism is a consequence of the ability of AKT to activate MDM2. Therefore, a reduction in AKT function (i.e., secondary to PTEN activation) activates p53 by removing the negative regulation imposed by MDM2. On the other hand, in situations of nutrient deprivation, AMPK-mediated phosphorylation of p53 increases its half-life and transcriptional activity.[48]

Epigenetic Modifications and Tumor Suppression

Evidence accumulated in the past decade has led to the realization that *epigenetic* changes affecting oncogenes and TSGs constitute important events contributing to the hallmarks of cancer. The cancer "epigenome" is characterized by global and gene-specific heritable modifications that affect gene expression but do not involve changes in DNA sequence. Three types of cancer-associated epigenetic modifications are currently recognized: DNA methylation, histone modifications, and micro-RNA (miRNA)-mediated gene silencing.[49] Although they are discussed separately in this chapter, we should emphasize that these are functionally interdependent mechanisms. For example, some miRNAs can modify the epigenetic landscape by regulating the expression of proteins involved in histone modifications or DNA methylation. Conversely, miRNA expression itself can be altered by DNA methylation or histone modifications (Figure 3-3 is a summary of the most relevant epigenetic changes observed in cancer cells).

DNA Methylation

DNA methylation is the covalent addition of a methyl group to the cytosine ring of a CpG dinucleotide. Although CpG dinucleotides are widely distributed throughout the genomes of eukaryotic organisms, CpG-rich regions (also called CpG islands) are particularly enriched in the promoter regions of genes. The silencing effect of DNA methylation can result from the direct inhibition of transcription factor binding to promoter regions or from the recruitment of repressive protein complexes to methylated regions. As explained later in this chapter, this latter mechanism often results in a more condensed, transcriptionally silent chromatin configuration.[49]

Genome-wide hypomethylation and promoter-specific hypermethylation are common features of cancer. These alterations can be detected in benign lesions and early-stage tumors, suggesting that they may precede classical genetic events. Global loss of DNA methylation at CpG islands was the first epigenetic alteration identified in cancers. In particular, hypomethylation at repetitive sequences in the genome is associated with genomic instability.[50,51] However, the most recognized epigenetic alteration in cancer cells is the promoter hypermethylation affecting TSGs. One of the first examples of this silencing mechanism was discovered in some *RB1* alleles associated with retinoblastoma.[50] Since then, hypermethylation as a mechanism of gene inactivation has been demonstrated for other TSGs, including *INK4a/p16* (*CDKN2A*), *MLH1*, *BRCA1*, *VHL* and *CDH1* (encoding E-cadherin). In many cases, collaboration between hypermethylation and genetic inactivation can also be documented. For example, promoter hypermethylation contributes to the inactivation of the wild-type allele of *CDKN2A* in colorectal cancer cells that have already lost one allele through deletion. That hypermethylation is causally involved in the repression of TSGs has been confirmed through the use of demethylating procedures. For example, acute elimination or inhibition of DNMT1 (a DNA methyltransferase) in colon cancer cells is sufficient to reactivate INK4a/p16 and induce cell cycle arrest.[51]

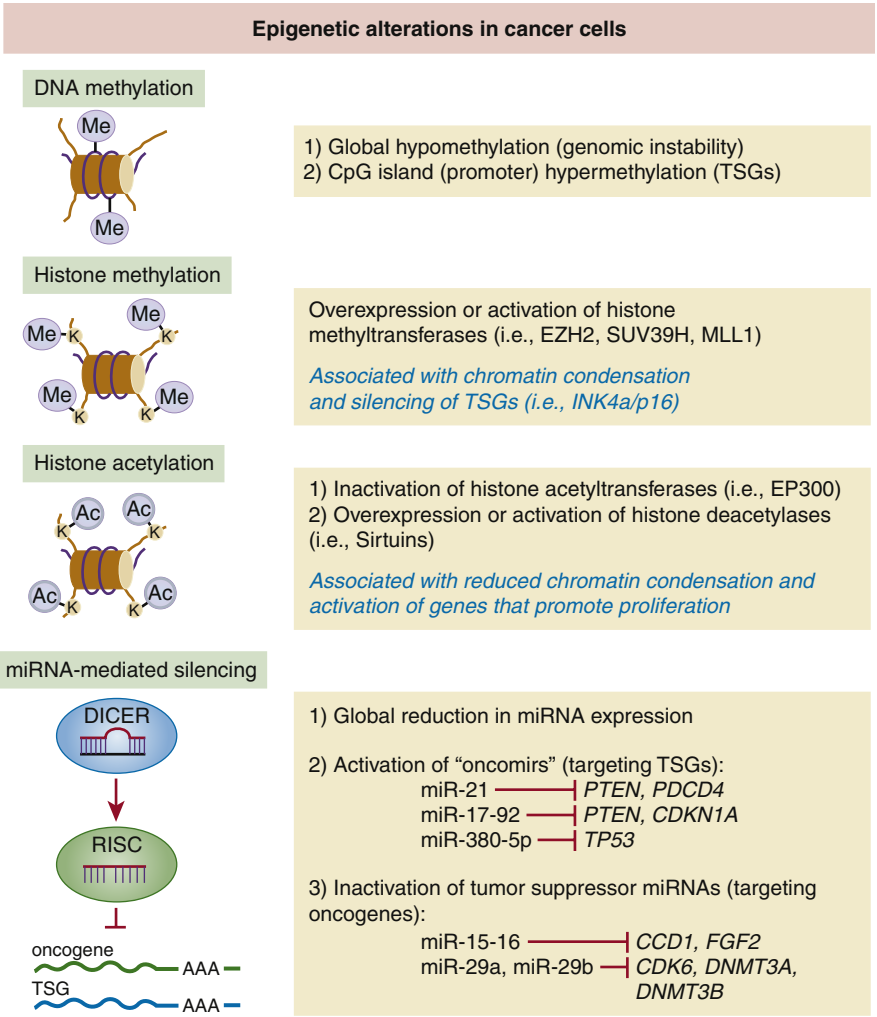


Figure 3-3 Epigenetic mechanisms involved in tumor suppression A brief summary of the main epigenetic changes affecting cancer cells.

Histone Modifications

The second group of cancer-associated epigenetic changes involves the covalent modification of histones, the proteins that form the core of nucleosomes. Among numerous modifications, acetylation and methylation of lysine (K) residues at the N termini of histones H3 and H4 are probably the best known. It has been proposed that the combinatorial addition or removal of acetyl and methyl groups to several K residues of H3 and H4 may serve as a "histone code" that dictates the degree of chromatin condensation and, therefore, the extent to which a genomic locus becomes transcriptionally active. For example, *trimethylation* of lysines 4, 36, or 79 and *acetylation* of lysines 9 or 14 of histone H3 are both associated with a relaxed chromatin configuration that facilitates transcription. Conversely, di- or trimethylation of lysine 9 or 27 of histone H3 is associated with a more condensed, transcriptionally silent chromatin configuration. It is important to keep in mind that the enzymatic complexes involved in these modifications (see later discussion) cooperate extensively with DNA methyltransferases (the enzymes that catalyze DNA methylation) to produce stable chromatin states.[51]

Although systematic analyses of histone modifications at the global level are still limited, some studies indicate that the disruption of normal patterns of *histone acetylation* can enhance tumorigenesis. For example, inactivation of two histone acetyltransferases (HATs), EP300 and CREBBP, by viral oncoproteins leads to a global reduction in acetylation of H3K18 (lysine 18 of histone H3), a modification that results in the transcriptional activation of a subset of genes that promote proliferation. Moreover, inactivating mutations of *EP300* have been reported in colorectal, gastric, breast, and pancreatic tumors. Similarly, the overexpression of histone deacetylases (HDACs) and *Sirtuins* (a class of HDAC) is also associated with cellular transformation, as observed in a variety of malignancies[51] (see Figure 3-3).

Finally, aberrant expression or activation of histone methyltransferases (HMTs) and histone demethylases (HDMs) is also associated with cancer. For example, overexpression of *EZH2* (an H3K27-specific HMT) is found in prostate, breast, and lung cancers. In prostate cancer, *EZH2* overexpression

correlates with H3K27 trimethylation and the silencing of TSGs. Similarly, the H3K9-specific methyltransferase SUV39H has been linked to tumor initiation and progression through silencing of the *CDKN2A* tumor suppressor locus in several cancers.[51] Perhaps the most paradigmatic and clinically relevant example of HMT activation is observed in leukemias affecting children. Translocation-mediated activation of *MLL1*, a gene encoding a H3K4 methyltransferase, has been reported in up to 80% of leukemias. Almost universally, translocations involving *MLL1* result in fusion proteins that function as constitutively active transcription factors, leading to the deregulation of several leukemia-promoting pathways.[49]

In contrast to HMTs, much less is known about the role of HDMs in tumorigenesis. So far, both overexpression and inactivation of HDMs seem to contribute to tumorigenesis.[51]

Micro-RNAs

The discovery of noncoding RNAs has unveiled unexpected complexities in the regulation of gene expression. As described here, these new regulatory factors can play significant roles during cancer initiation and progression by way of modulating classic oncogenic pathways.

Micro-RNAs (miRNAs) are small (18 to 25 nucleotides long), evolutionarily conserved, noncoding RNAs that control the translation and stability of protein-encoding messenger RNAs (mRNAs). miRNAs are produced through the sequential processing of primary transcripts (pri-miRNAs) by the ribonucleases Drosha and Dicer1. After processing, the guide RNA strand of a mature miRNA (which is complementary to a mRNA target) combines with *Argonaut* proteins to form an RNA-induced silencing complex (RISC), the final effector that targets mRNAs for cleavage or translational repression.[52]

High-throughput genetic screens have identified more than 1000 miRNA-encoding sequences in the human genome, which are typically arranged in large clusters expressed polycistronically. Together, they are predicted to control the expression of thousands of protein-encoding genes. Given their wide spectrum of functions, it is hardly surprising that aberrant miRNA expression contributes to tumorigenesis. Remarkably, although many miRNA-encoding sequences have been mapped to genomic regions that are amplified in human cancers, miRNA expression profiles reveal a global downregulation of miRNA expression in cancer cells. Although the mechanisms responsible for this phenomenon are not completely understood, it has been proposed that some oncoproteins (i.e., MYC) can directly contribute to the global silencing of miRNAs. Alternatively, cancer initiation may require the partial repression of miRNA biogenesis. In support of this idea, human tumors often show monoallelic deletions of *DICER1* and, accordingly, tumor formation is accelerated by monoallelic deletion of *DROSHA* or *DICER1* in mouse models.[52,53]

Deregulation of individual miRNAs can contribute to tumorigenesis by altering the expression of conventional oncogenes and TSGs. For example, "oncogenic miRNAs" (also known as *oncomirs*) negatively regulate the expression of TSGs, whereas "tumor suppressor miRNAs" downregulate the expression of oncogenes. Among oncomirs, *miR-21* was one of the first miRNAs found to be elevated in human cancers. In animal models, overexpression of *miR-21* enhances the tumorigenic effect of *K-Ras* in the lung in part by targeting *PTEN*. Similarly, amplification of the *miR-17~92* cluster has been documented in both solid and hematopoietic cancers. Genes targeted by individual miRNAs in this cluster include the tumor suppressors *PTEN*, *BIM*, and *CDKN1A*.[53,54]

The first tumor suppressor miRNA identified was *miR-15~16*, a cluster localized in a genomic region that is deleted in more than 50% of patients with B-cell chronic lymphocytic leukemia (B-CLL). Targets of this cluster include the oncogenes *CCD1*, *FGF2*, and *FGFR1*. Similarly, *miR-29a* and *miR-29b*, which are inactivated in mantle cell lymphoma, can target multiple cell cycle regulators and oncogenes.[52]

Given the relevance of the *TP53* tumor suppressor in human cancers, efforts to link p53 function to miRNA regulation have already led to important discoveries. For example, p53 induces several miRNAs, including *miR-34*, *miR-200*, and *miR-192*. Among these, the *miR-34* family represses genes that promote proliferation and survival, such as *CDK6* and *BCL2*. Conversely, *TP53* can be itself targeted by oncomirs in some cancers.[53]

It is worth mentioning that the extent to which an individual miRNA or miRNA cluster behaves as an oncomir or tumor suppressor is highly dependent on cellular context. Because a single miRNA can target multiple mRNAs, the differential expression of potential target mRNAs in different cell types means that the deregulation of a miRNA may lead to different outcomes depending on the cell type. For example, despite the fact that the *miR-29* family acts as a tumor suppressor in many tumors, it can behave as an oncomir in breast cancer, presumably because of the particular constellation of target mRNAs in breast cancer cells.

Conclusions

During the past 40 years, the exhaustive characterization of TSG products has provided us with a relatively robust conceptual framework to understand the genetic and epigenetic

bases of cancer. From the preceding sections, it should be clear that tumor suppressor proteins and cancer-associated micro-RNAs are involved in the control of almost any cellular function, and yet the disruption of their activities must somehow confer a proliferative advantage by influencing the decision of a cell to continue cycling. This feature helps explain the almost universal involvement of the pRB and p53 signaling networks in the pathogenesis of most human cancers. It is believed that a better understanding of this Achilles' heel of cancer will lead some day to more effective therapies. As data accumulate, however, it is becoming increasingly evident that the emergent picture calls for a much-needed contextualization of TSG functions. A full understanding of the different contexts in which (or against which) a TSG functions remains a daunting task for the future. So far, significant progress has been made in the identification of signaling or metabolic pathways disrupted by the loss of function of some TSGs. By comparison, we know much less about the relationships between various genetic alterations, or the relevance of their temporal appearance, for the tumorigenic outcome. On a different level, given the rather universal functions that most TSGs have in normal cells, it is remarkable that the inactivation of many TSGs leads to specific tumor types. Although we still do not understand the basis of tissue specificity, answers to this question will be most certainly provided by a better understanding of the connection among tumor suppression, tissue development, and processes that govern the differentiation and self-renewal of normal stem cells. Finally, advances in whole-genome sequencing are already revealing the existence of low-penetrance cancer susceptibility genes, which might play an important role as modifiers of cancer risk in human populations.

Key References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
2. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004;10:789-799.
3. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004;432:316-323.
4. Sherr CJ. Principles of tumor suppression. *Cell*. 2004;116:235-246.
5. Hoeijmakers JH. DNA damage, aging, and cancer. *N Engl J Med*. 2009;361:1475-1485.
6. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature*. 2001;411:366-374.
7. Steele RJ, Lane DP. P53 in cancer: a paradigm for modern management of cancer. *Surgeon*. 2005;3:197-205.
8. Berger AH, Knudson AG, Pandolfi PP. A continuum model for tumour suppression. *Nature*. 2011;476:163-169.
9. Fletcher O, Houlston RS. Architecture of inherited susceptibility to common cancer. *Nat Rev*. 2010;10:353-361.
19. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev*. 2008;8:671-682.
20. Knudsen ES, Knudsen KE. Tailoring to RB: tumour suppressor status and therapeutic response. *Nat Rev*. 2008;8:714-724.
21. Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev*. 2009;9:749-758.
22. Goh AM, Coffill CR, Lane DP. The role of mutant p53 in human cancer. *J Pathol*. 2011;223:116-126.
24. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*. 2007;39:165-167.
25. Anderson CA, Pettersson FH, Barrett JC, et al. Evaluating the effects of imputation on the power, coverage, and cost efficiency of genome-wide SNP platforms. *Am J Hum Genet*. 2008;83:112-119.
26. Hao K, Schadt EE, Storey JD. Calibrating the performance of SNP arrays for whole-genome association studies. *PLoS Genet*. 2008;4:e1000109.
27. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005;436:725-730.
28. Alimonti A, Carracedo A, Clohessy JG, et al. Subtle variations in Pten dose determine cancer susceptibility. *Nat Genet*. 2010;42:454-458.
29. Bellacosa A, Godwin AK, Peri S, et al. Altered gene expression in morphologically normal epithelial cells from heterozygous carriers of BRCA1 or BRCA2 mutations. *Cancer Prev Res (Phila)*. 2010;3:48-61.
32. Massague J. G1 cell-cycle control and cancer. *Nature*. 2004;432:298-306.
33. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev*. 2009;9:153-166.
35. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev*. 2003;13:77-83.
36. Larsson LG. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. *Semin Cancer Biol*. 2011;21(6):367-376.
37. Polager S, Ginsberg D. p53 and E2f: partners in life and death. *Nat Rev*. 2009;9:738-748.
38. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev*. 2010;24:2463-2479.
39. Feldser DM, Greider CW. Short telomeres limit tumor progression in vivo by inducing senescence. *Cancer Cell*. 2007;11:461-469.
40. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. Pro-senescence therapy for cancer treatment. *Nat Rev*. 2011;11:503-511.
41. Collado M, Serrano M. The power and the promise of oncogene-induced senescence markers. *Nat Rev*. 2006;6:472-476.
42. Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS. BRAF(E600) in benign and malignant human tumours. *Oncogene*. 2008;27:877-895.
43. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436:720-724.
44. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev*. 2009;23:537-548.
45. Sabatini DM. mTOR and cancer: insights into a complex relationship. *Nat Rev*. 2006;6:729-734.
46. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev*. 2009;9:550-562.
47. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev*. 2011;11:85-95.
48. Vousden KH, Ryan KM. p53 and metabolism. *Nat Rev*. 2009;9:691-700.
49. Taby R, Issa JP. Cancer epigenetics. *CA Cancer J Clin*. 2010;60:376-392.
50. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev*. 2004;4:143-153.
51. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. 2011;17:330-339.
52. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev*. 2006;6:857-866.
53. Lujambio A, Lowe SW. The microcosmos of cancer. *Nature*. 2012;482:347-355.
54. Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev*. 2011;11:849-864.

References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
2. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004;10:789-799.
3. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004;432: 316-323.
4. Sherr CJ. Principles of tumor suppression. *Cell*. 2004;116:235-246.
5. Hoeijmakers JH. DNA damage, aging, and cancer. *N Engl J Med*. 2009;361: 1475-1485.
6. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature*. 2001;411:366-374.
7. Steele RJ, Lane DP. P53 in cancer: a paradigm for modern management of cancer. *Surgeon*. 2005;3:197-205.
8. Berger AH, Knudson AG, Pandolfi PP. A continuum model for tumour suppression. *Nature*. 2011;476:163-169.
9. Fletcher O, Houlston RS. Architecture of inherited susceptibility to common cancer. *Nat Rev*. 2010;10:353-361.
10. Balmain A. Cancer genetics: from Boveri and Mendel to microarrays. *Nat Rev*. 2001;1:77-82.
11. Harris H, Miller OJ, Klein G, Worst P, Tachibana T. Suppression of malignancy by cell fusion. *Nature*. 1969;223:363-368.
12. Allderdice PW, Miller OJ, Miller DA, et al. Chromosome analysis of two related heteroploid mouse cell lines by quinacrine fluorescence. *J Cell Sci*. 1973;12:263-274.
13. Klein G, Bregula U, Wiener F, Harris H. The analysis of malignancy by cell fusion. I. Hybrids between tumour cells and L cell derivatives. *J Cell Sci*. 1971;8:659-672.
14. Fearon ER. Human cancer syndromes: clues to the origin and nature of cancer. *Science*. 1997;278:1043-1050.
15. Knudson AG. Two genetic hits (more or less) to cancer. *Nat Rev*. 2001;1: 157-162.
16. Cavenee WK, Dryja TP, Phillips RA, et al. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*. 1983;305:779-784.
17. Knudson Jr AG, Meadows AT, Nichols WW, Hill R. Chromosomal deletion and retinoblastoma. *N Engl J Med*. 1976;295:1120-1123.
18. Friend SH, Bernards R, Rogelj S, et al. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*. 1986;323:643-646.
19. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev*. 2008;8:671-682.
20. Knudsen ES, Knudsen KE. Tailoring to RB: tumour suppressor status and therapeutic response. *Nat Rev*. 2008;8:714-724.
21. Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev*. 2009;9:749-758.
22. Goh AM, Coffill CR, Lane DP. The role of mutant p53 in human cancer. *J Pathol*. 2011;223:116-126.
23. Meijers-Heijboer H, van den Ouweland A, Klijn J, et al. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet*. 2002;31:55-59.
24. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*. 2007;39: 165-167.
25. Anderson CA, Pettersson FH, Barrett JC, et al. Evaluating the effects of imputation on the power, coverage, and cost efficiency of genome-wide SNP platforms. *Am J Hum Genet*. 2008;83:112-119.
26. Hao K, Schadt EE, Storey JD. Calibrating the performance of SNP arrays for whole-genome association studies. *PLoS Genet*. 2008;4:e1000109.
27. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005;436:725-730.
28. Alimonti A, Carracedo A, Clohessy JG, et al. Subtle variations in Pten dose determine cancer susceptibility. *Nat Genet*. 2010;42:454-458.
29. Bellacosa A, Godwin AK, Peri S, et al. Altered gene expression in morphologically normal epithelial cells from heterozygous carriers of BRCA1 or BRCA2 mutations. *Cancer Prev Res (Phila)*. 2010;3:48-61.
30. Venkatachalam S, Shi YP, Jones SN, et al. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J*. 1998;17:4657-4667.
31. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. *Nat Rev*. 2001;1:222-231.
32. Massague J. G1 cell-cycle control and cancer. *Nature*. 2004;432:298-306.
33. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev*. 2009;9:153-166.
34. Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta*. 1998;1378:F115-F177.
35. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev*. 2003;13:77-83.
36. Larsson LG. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. *Semin Cancer Biol*. 2011;21(6):367-376.
37. Polager S, Ginsberg D. p53 and E2f: partners in life and death. *Nat Rev*. 2009;9:738-748.
38. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev*. 2010;24:2463-2479.
39. Feldser DM, Greider CW. Short telomeres limit tumor progression in vivo by inducing senescence. *Cancer Cell*. 2007;11:461-469.
40. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. Pro-senescence therapy for cancer treatment. *Nat Rev*. 2011;11:503-511.
41. Collado M, Serrano M. The power and the promise of oncogene-induced senescence markers. *Nat Rev*. 2006;6:472-476.
42. Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS. BRAF(E600) in benign and malignant human tumours. *Oncogene*. 2008;27:877-895.
43. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436:720-724.
44. Jones RG, Thompson CB. Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev*. 2009;23:537-548.
45. Sabatini DM. mTOR and cancer: insights into a complex relationship. *Nat Rev*. 2006;6:729-734.
46. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev*. 2009;9:550-562.
47. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev*. 2011;11:85-95.
48. Vousden KH, Ryan KM. p53 and metabolism. *Nat Rev*. 2009;9:691-700.
49. Taby R, Issa JP. Cancer epigenetics. *CA Cancer J Clin*. 2010;60:376-392.
50. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev*. 2004;4: 143-153.
51. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. 2011;17:330-339.
52. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev*. 2006;6:857-866.
53. Lujambio A, Lowe SW. The microcosmos of cancer. *Nature*. 2012;482:347-355.
54. Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev*. 2011;11:849-864.

Alan D. D'Andrea

4

DNA Repair Pathways and Human Cancer

Introduction

DNA repair is central to the field of cancer biology, and it has important implications for cancer diagnosis and treatment. Cancer cells are often deficient in a normal DNA repair function, and this deficiency allows the tumor to develop genomic instability.[1,2] With defective DNA repair, the tumor cell can break and reform chromosomes, generate new oncogenic fusion genes, disrupt tumor suppressor genes, amplify drug resistance genes, and progress to a more malignant state. A DNA repair deficiency also accounts for the enhanced sensitivity of tumor cells to genotoxic agents, such as ionizing radiation (IR) and genotoxic chemotherapy. A specific defect in homologous recombination DNA repair also renders a tumor cell hypersensitive to polyADP ribose polymerase (PARP) inhibitors. A thorough knowledge of DNA repair mechanisms in normal and cancer cells may therefore lead to better clinical management of cancer.

The Spectrum of DNA Damage

Spontaneous DNA Damage

In order to understand the process of DNA repair, one must first consider the wide range of DNA-damaging events in a cell. DNA may undergo spontaneous damage, such as deamination of cytosine or spontaneous hydrolysis of the phosphodiester backbone. DNA may develop mismatched bases, perhaps resulting from the deployment of an error-prone DNA polymerase during S phase progression. DNA may be attacked by reactive oxygen species (ROS). Indeed, some of the most sophisticated DNA repair mechanisms in a cell are mechanisms that cope with the removal of oxidative DNA lesions.

Of particular relevance to cancer is the DNA damage from alkylating agents or from ultraviolet (UV) light or IR. DNA damage resulting from these environmental agents can lead to heightened mutagenesis and oncogenesis. Also, many of these agents themselves have anticancer activity. Thus, DNA-damaging agents can cause human cancer but, ironically, are among the primary means available to clinicians for treating cancer. Accordingly, some chemotherapeutic agents have effective anticancer activity in the short run but are responsible for causing secondary cancers in the long run.

DNA Damage from Antineoplastic Therapeutic Agents

Most anticancer agents function by directly damaging DNA. Effective anticancer drugs include monofunctional alkylating agents (cyclophosphamide, BCNU), bifunctional alkylating agents, (cisplatin, carboplatin, oxaliplatin), and DNA intercalating agents (adriamycin). In addition, IR and the radiomimetic agent bleomycin can cause double-strand breaks in DNA directly. Bleomycin is a small glycopeptide that chelates ferrous ion and binds to specific sequences of double-stranded DNA containing pyrimidine repeats. In the presence of oxygen, bleomycin generates a local high concentration of hydroxyl radicals capable of causing local double-strand breaks. Other drugs, such as the topoisomerase inhibitors etoposide and camptothecin, can lead to the accumulation of DNA damage. Thus, known anticancer agents can generate a wide range of DNA damage, including damaged bases, single-strand breaks, and double-strand breaks.

It is important for oncologists to bear in mind that anticancer drugs generate their cytotoxic effects through DNA damage. First, effective anticancer protocols often include a combination of chemotherapeutic agents and IR. Together, these agents cause a broader spectrum of DNA damage in the tumor than single agent therapy (monotherapy). This broad spectrum may contribute to the synergy observed with these agents. Second, chemotherapy combinations are often chosen to limit toxicity to normal tissue. Agents that generate the same class of DNA damage (such as IR and bleomycin, which both generate double-strand breaks) may have enhanced toxicity compared to other combinations.

Some newer classes of drugs inhibit normal DNA repair processes. These so-called chemosensitizers may be particularly effective when used in combination with a more traditional cytotoxic, DNA-damaging drug. A combination of a DNA repair inhibitor and a direct DNA damaging agent can also result in significant toxicity to normal tissue. One way to limit this toxicity would be to deliver one agent such as the chemosensitizer systemically but to deliver the other agent, such as IR, locally to the tumor.

DNA Repair

DNA repair is strictly defined as the cellular responses that are associated with the restoration of the normal base pair sequence and structure of damaged DNA. As described in the following section, there are six primary DNA repair pathways, and each pathway is composed of a series of biochemical events leading to the sensing, excision, and restoration of the normal DNA sequence.

The Systematic Study of DNA Repair

It is instructive to consider the history of DNA repair research as it relates to cancer biology. Early studies of DNA repair evolved from the study of normal DNA replication and metabolism. These early studies relied heavily on the use of damaged DNA templates as substrates for the purification of DNA repair enzymes. Such templates were incubated with cell-free extracts, and the recovered DNA was analyzed for specific incision and excision events. Not surprisingly, these assays uncovered many of the pertinent endonuclease and exonuclease activities required for DNA repair. It has become increasingly apparent that DNA repair proteins are assembled in protein/protein complexes, such as the excision repair complex or the mismatch repair complex. Still, the regulatory networks and relevant posttranslational modifications of DNA repair proteins (i.e., phosphorylations and ubiquitinations) were largely missed by these early biochemical studies.

The study of inherited human DNA repair disorders also contributed greatly to the recognition of the six major DNA repair pathways (see later discussion). These studies depended on the establishment of mutant human cell lines derived from patients with genetic diseases. For instance, in 1968, James Cleaver isolated fibroblast lines from humans with the disease xeroderma pigmentosum (XP).[3,4] Importantly, these lines retained their UV light–hypersensitivity phenotype and have been invaluable tools for somatic cell fusion, complementation analysis, and expression cloning of XP genes. Subsequently, other investigators were able to establish mutant cell lines from humans with other DNA repair disorders such as ataxia-telangiectasis (A-T), Fanconi anemia (FA), and Nijmegen breakage syndrome (NBS).[5] These cell lines continue to be used extensively as models of human cancers that also lack the relevant DNA repair pathways.

The study of model organisms has contributed greatly to our understanding of DNA repair processes. For instance, investigators isolated mutants in the yeast *Saccharomyces cerevisiae*, which were hypersensitive to UV light, IR, or DNA crosslinking agents. In many cases, the genes that were mutated in these yeast strains cooperated in common DNA repair and DNA damage response pathways. IR-induced double-strand breaks in DNA are normally repaired by the DNA repair process of homologous recombination (HR). Accordingly, many of the relevant genes corresponding to these mutant strains and required for normal HR repair were first isolated in yeast. Thereafter, the human homologues of these genes were identified. Other model organisms and cell lines have been especially important in the identification of genes involved in DNA repair pathways, such as mismatch repair[6] and translesion DNA synthesis.[7] Among the most useful model systems for studying DNA repair are the *Caenorhabditis elegans*[8] and chicken (DT40) genetic systems.[9]

In the postgenomic era, and following the identification of a large number (perhaps 130) of distinct DNA repair proteins,[10] investigators have turned to x-ray crystallography for a detailed understanding of DNA repair protein interaction with damaged DNA. The structures of many endonucleases, helicases, and ligases are now available, providing the opportunity for computer-assisted drug development (CADD) of DNA repair enzyme inhibitors. Also, mass spectrometry has been used to identify critical posttranslational modifications of DNA repair proteins. These modifications appear to be essential to the proper localization and assembly of DNA repair complexes around sites of DNA damage. The modifications may also regulate the intrinsic catalytic activity of the repair complexes. These protein modifications can be used as surrogate markers, or biomarkers, of DNA repair activity in a given tumor type as well (see later discussion).

The Six Major DNA Repair Pathways in Human Cells

As described previously, the combination of (1) biochemistry with damaged DNA templates, (2) human mutant cell lines with genetic deficiencies of DNA repair, (3) genetics of yeast mutants with IR or UV sensitivity, and (4) structural studies of DNA repair proteins has led to the establishment of six major DNA repair pathways. These pathways

Table 4-1 The Six Major DNA Repair Pathways

DNA Damage Repair Pathway	Function	Examples of Gene Mutation	Examples of Altered Expression of a Normal Gene	Effect of Loss of Pathway on Clinical Response
Base-excision repair (BER)	Repair of damaged bases or single-strand DNA breaks	None reported	None reported	None reported
Mismatch repair (MMR)	Repair of mispaired nucleotides	Mutation of *MSH2*, *MSH6*, and *MLH1* in Turcot syndrome (brain and colon tumors) and *HNPCC* (colon and gynecologic cancers)	Loss of expression of *MSH2* or *MLH1* in sporadic colon cancer	Resistance to DNA monoadducts Sensitivity to DNA crosslinks
Nucleotide-excision repair (NER)	Excision of a variety of helix-distorting DNA lesions	Mutation of *XPA*, *XPB*, *XPC*, *XPE*, *XPF*, or *XPG* in xeroderma pigmentosum (skin cancer) Variant expression of *ERCC1* or *XPD* in lung cancer	Loss of *XPA* expression in testicular germ-cell tumors	Sensitivity to DNA adducts
Homologous recombination (HR)	Repair of double-strand DNA breaks	*BRCA1/2* mutated in early-onset breast/ovarian, prostate, pancreas, and gastric cancers *FANC* genes mutated in Fanconi anemia	Loss of expression of *BRCA1/2* in ovarian and lung cancers Loss of *NBS1* expression in prostate cancer	Sensitivity to DNA double-strand breaks
Nonhomologous end joining (NHEJ)	Repair of double-strand DNA breaks	DNA ligase IV mutated in Lig4 syndrome (leukemia) Artemis mutated in Omenn syndrome (lymphoma)	Loss of *Ku70* expression in cervical, rectal, and colon cancers Loss of *Ku86* expression in rectal cancer	Sensitivity to DNA double-strand breaks
Translesional synthesis (TLS)	Bypass of DNA adducts during DNA replication	DNA pol E mutated in xeroderma pigmentosum variant (XPV; skin cancers)	Pol β overexpressed in uterus, ovary, prostate, and stomach cancers Pol iota overexpressed in breast cancer	Resistance to DNA adducts

are base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), homologous recombination (HR), nonhomologous end joining (NHEJ), and translesion DNA synthesis (TLS) (Table 4-1).

There is also considerable redundancy in the function of the DNA repair pathways. When one pathway is disrupted, another pathway can partially compensate, especially if the second pathway is upregulated. For instance, a cell that is deficient in HR repair may depend more on the error-prone NHEJ repair pathway for the repair of double-strand breaks. Also, thymine dimers, which are generated by UV light exposure, can be repaired by NER repair or bypassed and effectively ignored by TLS polymerases. In some cases, the absence of one DNA repair pathway results in a hyperdependence on one or more other DNA repair pathways.[11,12] This so-called synthetic lethality among DNA repair pathways has important implications for the design of new anticancer drugs (see later discussion).

The six DNA repair pathways are not constitutively activated, but instead are highly regulated. The pathways are often activated at discrete times in the cell cycle. For instance, HR repair and TLS repair are active during the S phase of the cell cycle. Also, the DNA repair pathways are differentially active in various tissues and cell types. For instance, HR and TLS are more active in rapidly growing cells, such as hematopoietic cells, whereas NHEJ is more active in postreplicative cells. Accordingly, absence of a particular DNA repair pathway may be particularly disruptive to the growth and survival of some normal tissues and some cancers. Here is a brief description of the six DNA repair pathways, with an emphasis on the enzymes in the pathways and the preference for DNA lesions repaired.

Base-Excision Repair

BER has been reviewed by Wilson.[13] The cell uses BER to correct damaged DNA bases or single-strand DNA breaks. These lesions often result from spontaneous DNA damage (DNA deamination or hydroxylation of bases) or from exposure to environmental alkylating agents. In this pathway, damaged bases are removed by one of at least 10 DNA glycosylases. The resulting apurinic/apyrimidinic (AP) sites are processed first by the Ape1 AP endonuclease, leaving a 5′ deoxyribose phosphate; then by an AP lyase activity, leaving a 3′-elimination product. Single-strand breaks are then filled in by a DNA polymerase, either with a single nucleotide or with a longer repair patch, followed by ligation. The latter events in the BER pathway are regulated by the enzyme PARP1 (polyADP ribose polymerase 1). During the DNA

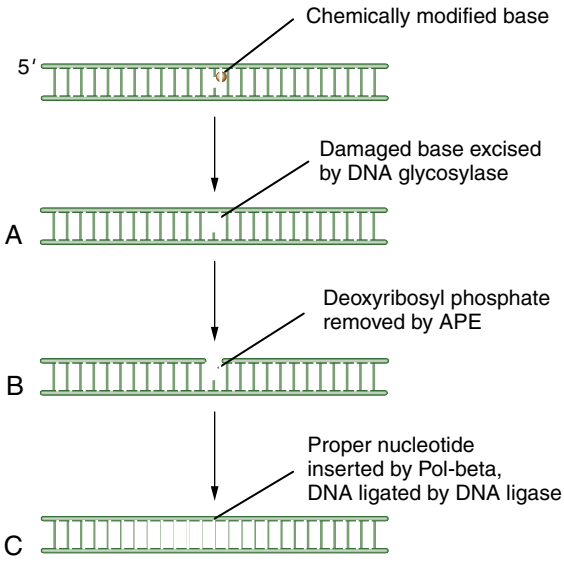


Figure 4-1 Schematic description of base-excision repair (BER) BER is focused on small DNA lesions, often from endogenous sources, resulting in minor helix distortions. Initially, the lesion is recognized by one of the cellular DNA glycosylases, which cleaves the covalent bond between the abnormal base and the deoxyribose sugar **(A)**. This cleavage leaves a so-called apurinic (A-P) site. Next, the apurinic endonuclease *(APE)* is recruited to cleave the phosphodiester backbone of the DNA **(B)**. Finally, an error-free polymerase, Pol-beta, is engaged to replace the normal nucleotide, followed by DNA ligation **(C)** and restoration of the normal double-stranded DNA sequence.

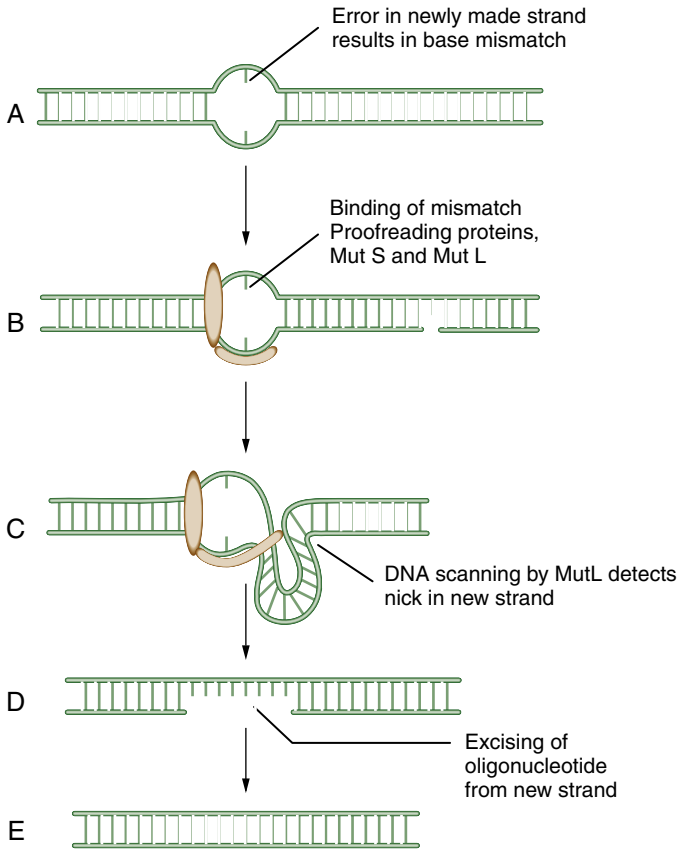


Figure 4-2 Schematic model of mismatch repair (MMR) Mismatch repair proteins function by sensing, binding, and repairing mistakes made during DNA replication. These mistakes include misincorporated bases and errors made during replication of microsatellite sequences **(A)**. MutS can bind to the mismatch **(B)** and generate a kink in the DNA **(C)**. This allows MutL to scan the DNA for a nearby single-strand nick in the newly replicated DNA. MutL then identifies, cleaves, and removes an oligonucleotide patch from the newly replicated strand **(D,E)**. This allows replication and the insertion of the proper DNA base at the site of the former mismatch. Mutations in the human genes encoding homologues of these bacterial proteins play a critical role in the inherited disease hereditary nonpolyposis colon cancer (HNPCC).

damage response, the PARP1 enzyme polyADP ribosylates BER enzymes and enhances BER activity. Accordingly, tumors that have high levels of BER activity may be hypersensitive to PARP inhibitors.[14] A schematic representation of BER is shown in Figure 4-1.

Mismatch Repair (MMR)

MMR has been reviewed by Modrich.[6] MMR rapidly removes mispaired nucleotides that result from replication errors and is also involved in the detection and repair of DNA adducts such as those resulting from platinum-based chemotherapeutic agents. Initially, the heterodimeric MSH complex recognizes the nucleotide mismatch, followed by its interaction with MLH1/PMS2 and MLH1/MLH3 complexes. Several proteins participate in the process of nucleotide excision and resynthesis. Tumor cells deficient in MMR have much higher mutation frequencies than normal cells and exhibit microsatellite instability, a genomic biomarker of the underlying defect. Patients with the genetic disease HNPCC (hereditary nonpolyposis colon cancer) have germline mutations of MMR genes and are predisposed to MMR-deficient colon cancers. Recent studies suggest that MMR-deficient cells may be hypersensitive to inhibitors of various DNA polymerases, such as POLB and POLG.[15] At least six genes—MSH2, MLH1, PMS2, MSH3, MSH6, and MLH3—are involved in mismatch repair. A schematic representation of MMR is shown in Figure 4-2.

Nucleotide-Excision Repair (NER)

NER acts on a variety of helix-distorting DNA lesions, caused mostly by exogenous sources that interfere with normal base pairing. This pathway may be particularly important in the response to adduct-forming chemotherapeutic agents such as platinum-based chemotherapy.[16] The primary function of NER appears to be the removal of damage such as pyrimidine dimers, which are induced by UV light. Members of the NER pathway include the XPA, XPB, XPC, XPD, XPE, and XPG proteins. Two other NER proteins, XPF and ERCC1, are especially important for the

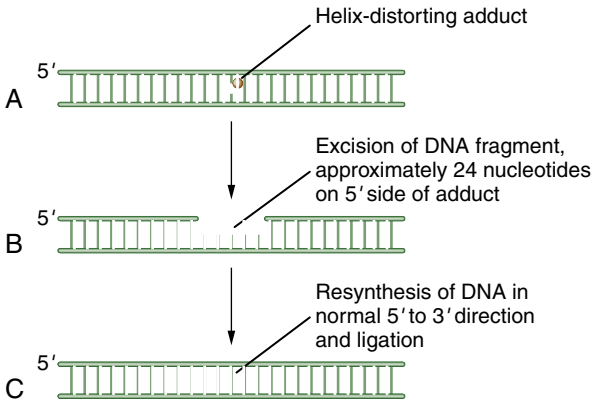


Figure 4-3 Schematic model of nucleotide excision repair (NER) NER is invoked when a base is modified by a larger helix-distorting lesion **(A)**, such as a UV-generated thymine dimer. Initially, the bulky lesion is recognized by a sensor complex, including the XPE protein (also known as DDB2). This protein is part of a ubiquitin conjugating complex, containing Cul4A and DDB1. The complex polyubiquitinates XPC, allowing for the recruitment of the excision repair complex. Next, a patch of nucleotides is excised from the damaged DNA. In general, the excision occurs approximately 24 nucleotides 5′ to the damaged base and 3 nucleotides to the 3′ side **(B)**. Finally, new DNA polymerization can occur, and the repaired DNA is ligated **(C)**. The nucleotide excision repair complex is a large multisubunit complex. Mutations in genes encoding subunits of this complex underlie the human disease xeroderma pigmentosum (XP). The complex also contains proteins that can recognize and remove bases with large bulky adducts, such as those generated by polycyclic hydrocarbons and aflatoxin B1.

processing of DNA crosslink repair. Recent studies indicate that monitoring the levels of these proteins in tumors may provide important biomarkers for predicting crosslinker drug sensitivity. For instance, some non–small-cell lung cancers are deficient in ERCC1, and this deficiency correlates with the cisplatin sensitivity of the specific tumor.[17]

As for the other DNA repair pathways, these proteins cooperate to recognize and excise the damaged nucleotides and resynthesize and ligate the damaged DNA strand. In the process of NER, initially a DNA-binding component, the DDB, binds to sites of damaged DNA, such as cyclopyrimidine dimers or 6-4 photoproducts. The DDB consists of DDB1 and DDB2. Mutations in the DDB2 gene cause the genetic complementation group XPE. DDB is part of a ubiquitin E3 ligase that polyubiquitinates XPC. Polyubiquitination of XPC enhances its DNA binding. This binding sets the stage for the downstream binding of the entire excision repair complex, TFIIH, thus leading to excision of the damaged bases.

Eukaryotic NER includes two major branches, transcription-coupled repair (TCR) and global genome repair (GGR). GGR is a slow, random process of inspecting the entire genome for injuries, whereas TCR is highly specific and efficient and concentrates on damage-blocking RNA polymerase II. The two mechanisms differ in substrate specificity and recognition, and hence the enzymes involved are important nodal points for posttranslational modifications. A schematic representation of NER is shown in Figure 4-3.

Homologous Recombination Repair

DNA double-strand breaks (DSBs) can be caused by many different environmental factors, including reactive oxygen species, IR, and certain antineoplastic drugs, such as bleomycin, anthracyclines, and topoisomerase inhibitors. Alternatively, DSBs can result from endogenous factors, especially during normal S-phase progression. Failure to repair DSBs can lead to a number of consequences, including mutations, gross chromosomal rearrangements, and other aberrations and eventually cell death. HR is a process by which DSBs are repaired through the alignment of homologous sequences of DNA and occurs primarily during the late S to M phase of the cell cycle. Initially the RAD50, MRE11, and NBS1 complex, which possesses a 3′-5′ exonuclease activity, exposes the 3′ ends on either side of the DSB, a process that may also require BRCA1. The 3′ advancing strand from the damaged chromosome then invades the complementary sequence of the homologous chromosome. The breast cancer susceptibility protein BRCA2 and the single-strand DNA binding protein RAD51 are required for the process. The 3′ end of this strand is then extended by an HR polymerase, by reading off of this complementary sequence. After replication has extended past the region of the DSB, the 3′ end of the advancing strand returns to the original chromosome, and replication continues. A schematic representation of HR is shown in Figure 4-4. HR repair is especially important in the repair of DSBs and DNA interstrand crosslinks. Because some tumors, particularly breast and ovarian tumors, are defective in HR repair, drugs that cause these lesions may be particularly effective in this setting.

Nonhomologous End Joining (NHEJ)

NHEJ, which has been reviewed by Lieber et al.,[18] is another major pathway for repairing DSBs. In common with HR, this pathway is important in the repair of agents that result in DSBs such as IR, bleomycin, topoisomerase II poisons, and anthracyclines. The DNA-dependent protein kinase (DNA-PK) consists of the catalytic subunit (DNA-PKcs) and the regulatory subunit (the Ku70/Ku80 heterodimer). The DNA-PKcs subunit is a serine/threonine kinase that belongs to the phosphatidylinositol-3 kinase family. The Ku80/Ku70 heterodimer (Ku) exhibits sequence-independent affinity for double-stranded termini and, on binding to DNA, recruits and activates the DNA-PKcs catalytic subunit.

Additional proteins are required for the completion of NHEJ, including the Artemis protein and DNA ligase IV. Importantly, NHEJ is an error-prone repair pathway. Because the process does not use a complementary template, the fusion of the blunt-ended DNA duplexes may result in

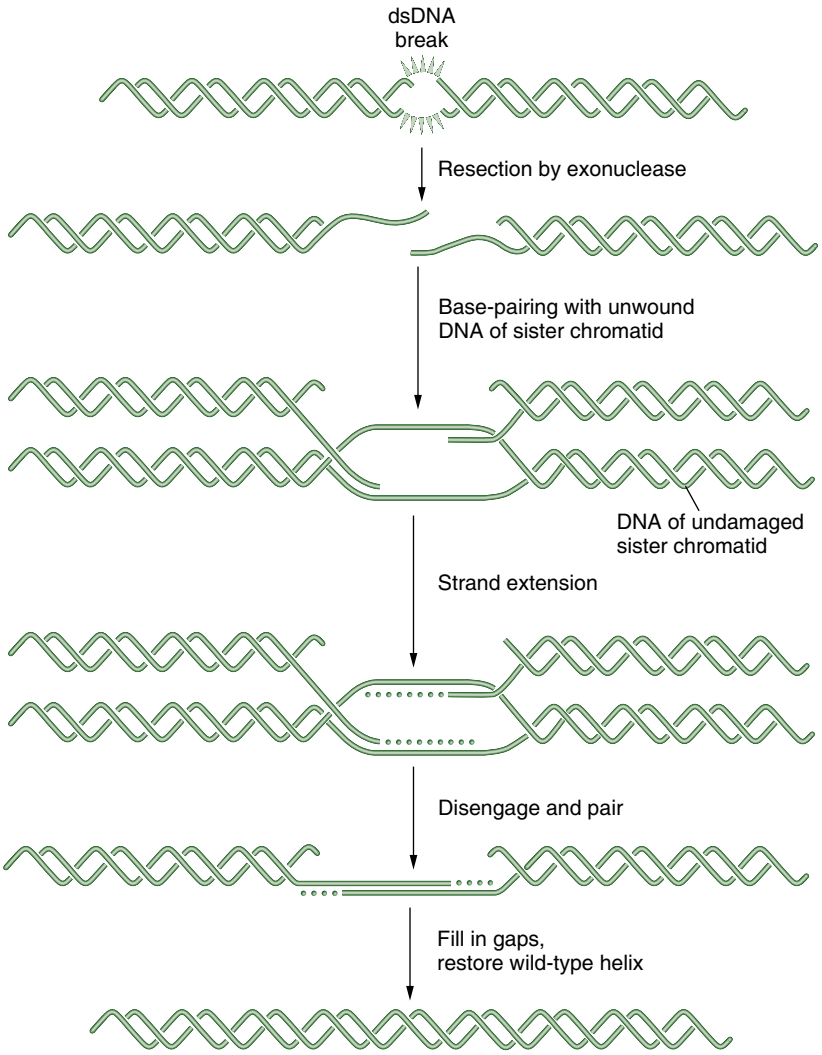


Figure 4-4 Schematic representation of homologous recombination (HR) HR repair is required for the normal repair of double-strand breaks (DSBs) as well as covalent interstrand DNA crosslinks. Initially, the DSB is recognized by a sensor. Some tumors have defects in HR repair, such as BRCA1 or BRCA2-deficient breast cancers. These tumor cells have prolonged time periods with unrepaired DSBs, thus leading to chromosome translocation events and a more malignant phenotype. Alternatively, the defective HR repair results in hyperdependence on the more error-prone NHEJ mechanism.

deletion or insertion of base pairs. A schematic representation of NHEJ is shown in Figure 4-5. NHEJ has a normal function in immune cells to generate diversity at the immunoglobulin and T-cell receptor gene loci.

Translesion DNA Synthesis (TLS)

The process of TLS is another mechanism for dealing with thymine dimers and bases with bulky chemical adducts. At a DNA replication fork, DNA adducts may cause a replicative polymerase such as DNA polymerase delta to stall. Cells have, therefore, developed sophisticated mechanisms for switching off the replicative polymerase and switching on alternative polymerases (i.e., a polymerase such as pol eta, which will replicate past certain DNA lesions with high fidelity).[19] Interestingly, human cells have at least 15 DNA polymerases, although the situations and mechanisms of their deployment are largely unknown.[20] Cancer may have a heightened dependence on one of the error-prone TLS polymerases, such as polymerases β or kappa, accounting for high rates of mutagenesis. A schematic representation of TLS is shown in Figure 4-6.

Examples of Redundancy among the DNA Repair Pathways

Specific DNA repair pathways can antagonize the activity of anticancer agents. The status of a particular DNA repair pathway in a tumor may therefore predict the best antitumor therapy. As described earlier, at least two DNA repair pathways, BER and NER, are dedicated to the removal of DNA bases modified by monofunctional alkylating agents. BER can cleave the bond linking the modified base to the deoxyribose. NER, in contrast, will remove the entire modified nucleotide, along with a small stretch of surrounding nucleotides. In either case, the undamaged DNA can be used to synthesize the normal DNA sequence, followed by ligation of the segments.

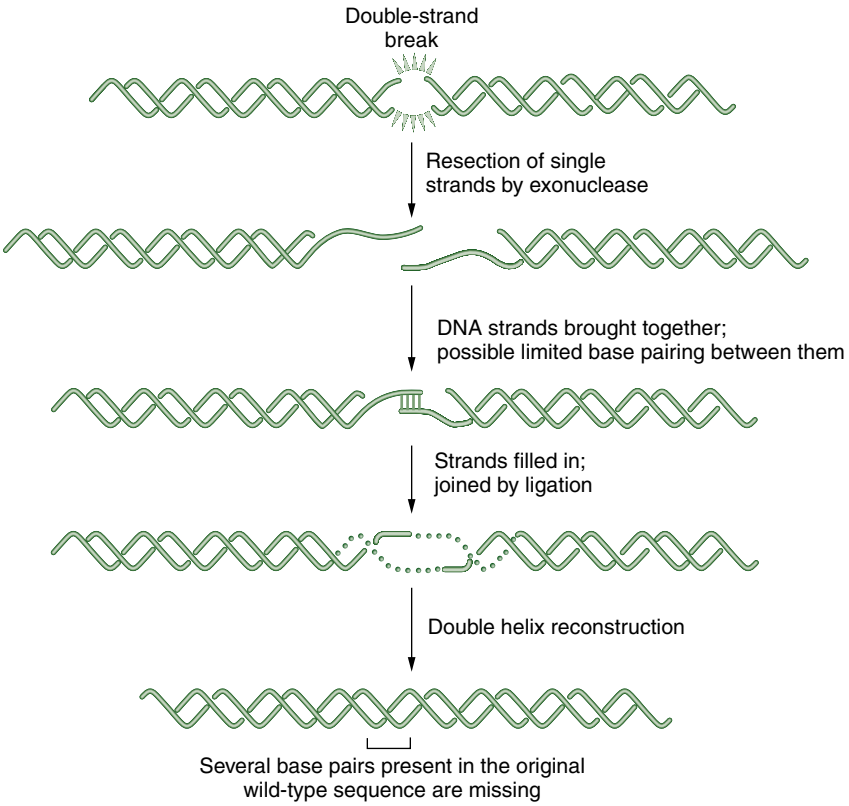


Figure 4-5 Schematic representation of nonhomologous end joining (NHEJ) NHEJ is an error-prone alternative to HR repair that can also be employed to repair double strand breaks. Since NHEJ does not use a homologous DNA template, such as a sister chromatid or a homologous chromosome, it often results in the insertion or deletion of new nucleotides at the fused DSB junction. In NHEJ, the DSBs are coated by the blunt end binding protein Ku. In some cases, the blunt ends may be brought together by limited microsequence homology. The enzymes DNA-PK, XRCC4, Artemis, and DNA ligase IV are required for the successful religation of the free ends. Interestingly, NHEJ appears to be the repair mechanism used for the cleavage and religation of immunoglobulin gene variable regions; thus the error-prone religation adds to the diversity of the somatically generated Ig gene repertoire. Germline mutations in some NHEJ genes, such as DNA-PK and Artemis, result in an inherited defect in NHEJ and a severe combined immunodeficiency syndrome.

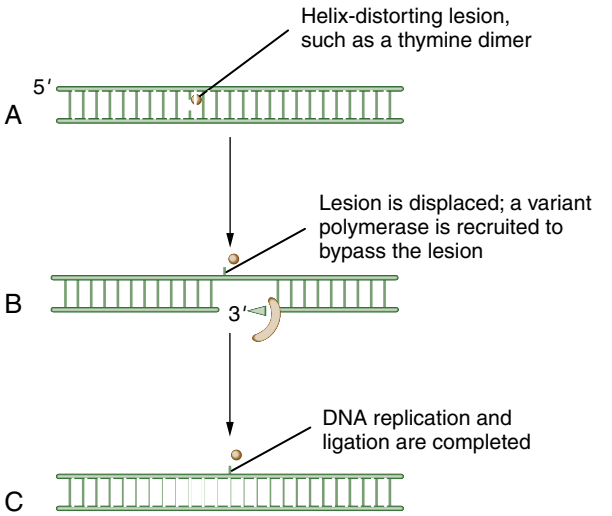


Figure 4-6 Schematic model of translesion DNA synthesis (TLS) repair TLS is not a DNA repair pathway per se; it is instead a mechanism of DNA damage bypass. In this process, an advancing replication fork encounters a damaged DNA base **(A)**. Although the replicative polymerase (the Pol delta complex) cannot read through the damaged base, a variant polymerase such as Pol eta can bypass the lesion. Cells have developed sophisticated mechanisms for switching polymerases **(B)**. For instance, in response to UV damage and the generation of a CPD (cyclopyrimidine dimer), the processivity factor, PCNA, becomes monoubiquitinated by RAD18. Modified PCNA now excludes Pol delta binding and has preferred binding for Pol eta. Pol eta is recruited, and it has the ability to "read through" the damaged base and insert the proper nucleotide (i.e., AA residues are replaced opposite the TT residues of a thymine dimer). Less is known about the regulation of TLS than about other DNA repair pathways. Depending on the kind of DNA damage, it is becoming increasingly clear that there are biochemical "switching" mechanisms for recruiting one of the other 12 TLS polymerases, as needed.

Cancer cells have other mechanisms for coping with modified bases. One enzyme, MGMT (0-6 methylguanine DNA methyltransferase), is capable of catalyzing the reversal of the chemical modification. Interestingly, this enzyme is switched off by MGMT gene promoter methylation in some solid tumors (gliomas and colorectal tumors), accounting, at least in part, for the hypersensitivity of these tumors to some monofunctional alkylating agents such as temozolamide.[21]

In addition, damaged bases in the DNA can be bypassed through the use of TLS. Through this mechanism, the modified lesions are sensed, the normal replicative polymerase is removed from the replication fork, and a new polymerase is invoked to bypass the lesions. Rapid TLS (damage avoidance) is essential in order for a cell to transverse S phase rapidly, without succumbing to replication arrest and apoptosis. Interestingly, however, TLS is an error-prone process, and the promiscuous use of TLS by cancer cells may result in their increased mutation frequency (see later discussion).

Some of the 15 variant polymerases can extend a nascent DNA strand past a thymine dimer or past a bulky DNA lesion. Other variant polymerases can replace a single nucleotide at the site of an unpaired base. One of these variant polymerases, referred to as POLeta, is mutated in the autosomal recessive human disease XP-variant (xeroderma pigmentosum variant). Absence of the POLeta enzyme results in UV light hypersensitivity, an inability to replicate

past thymine dimers, and a predisposition to squamous cell cancers.

The variant polymerases exhibit a variable level of fidelity. Important unanswered questions in the TLS research field include the following: (1) What are the circumstances and mechanisms for recruiting the variant polymerase to a specific damaged DNA site? and (2) Are any of these variant polymerases overexpressed or dysregulated in cancer, accounting for the elevated mutation frequency of solid tumors?

As described previously, IR causes double-strand breaks as well as a wide range of oxidative DNA damage. Two redundant DNA repair pathways, HR repair and NHEJ repair, are particularly adept at dealing with double-strand break damage in a cancer cell. In clinical oncology, some tumors that have defects in these DNA repair processes are particularly sensitive to the cytolytic activity of IR. Also, radiation resistance can emerge through the induction of these DNA repair activities in treated tumor cells. Tumor cells that grow in a more hypoxic environment may also be more resistant to the killing effect of IR, perhaps because of the decrease in oxidative damage generated in these cells.

Regulation of the Six DNA Repair Pathways

As described earlier, the major proteins involved with DNA repair include sensory (DNA binding) proteins, enzymes that remove damaged bases, and enzymes that restore the normal DNA sequence. A large number of regulatory enzymes also control each DNA repair pathway. These enzymes are required for switching DNA repair on and off, as needed by the cells. Regulatory enzymes, such as helicases, serve to load DNA repair complexes at the sites of DNA damage. Other regulatory enzymes, such as topoisomerases, serve to unwind damaged DNA, in order to facilitate DNA repair complex assembly, loading into chromatin, and disassembly.

A major subclass of regulatory enzymes add critical posttranslational modifications to DNA repair enzymes. For instance, in BER, a sumoylating enzyme modifies one of the glycosylases, TDG, thereby enhancing the activity of the glycosylase in removing damages bases.[22,23] In NER, an E3 ligase complex (Cul4A, DDB1, DDB2) activates the polyubiquitination of the XPC protein. This XPC modification is a necessary event for the downstream activity of the nucleotide excision repair complex.[24] In TLS, an E3 ligase, RAD18, monoubiquitinates the DNA processivity factor, PCNA, and allows this clamp to interact with the downstream DNA polymerase Pol eta. Many of these regulatory processes have recently been reviewed.[25]

Regulatory enzymes are also required to disassemble DNA repair enzymes after a repair pathway has been completed. For instance, the negative regulatory phosphatase, PP2A, removes phosphate from ATM substrates and thereby switches off the DNA damage response.[26] The deubiquitinating enzyme, USP1, can remove ubiquitin from activated FANCD2 and thereby switch off HR repair.[27] USP1 can also deubiquitinate PCNA and switch off TLS repair.[28,29] These negative regulatory events have also recently been reviewed.[25] The function of these regulatory enzymes underscores the dynamic nature of DNA repair. Loss of these regulatory mechanisms may result in the failure to (1) activate an error-free DNA repair pathway or (2) inactivate an error-prone DNA repair pathway. In either case, the consequence may be a heightened mutation frequency of the dysregulated cell and a predisposition to cancer.

Recent studies, generated through human cancer genome sequencing projects,[30] have demonstrated that some tumors, primarily melanomas and squamous cell lung cancers, have elevated levels of random point mutagenesis. This elevated point mutation signature indicates that these tumors (1) may have an underlying dysregulation of DNA repair and (2) may be more sensitive to specific therapies (see later discussion of synthetic lethality relationships). Finally, the regulation of DNA repair is a major focus of the DNA repair research field. For instance, it is unknown how DNA repair pathways are activated in specific cell types or at specific stages of the cell cycle. Because some DNA repair processes, such as HR repair, are specifically activated in S phase, it is likely that these pathways are activated by the cdk family of cyclin-dependent kinases. Several recent studies have performed shRNA screens to identify other regulatory genes that control DNA repair.[31-33] Many of these genes encode proteins that control the posttranslational modification of known DNA repair proteins or that regulate chromatin disassembly. Disruption of these gene products with shRNAs or small molecules can block DNA repair and further sensitize tumors to conventional cytotoxic cancer therapies.

Sequential Use of Three DNA Repair Pathways to Repair DNA Crosslinks

Interstrand DNA crosslinks (ICLs) make up a particular subtype of DNA lesions, and these lesions have an especially potent biological effect. Because ICLs involve the covalent modification of both strands of DNA, the lesions can prevent DNA strand separation during DNA replication. The lesions can also prevent the access of some DNA repair enzymes and transcription factors that normally require DNA strand separation for DNA binding to occur. DNA crosslinking agents, such as cisplatin derivatives (carboplatin

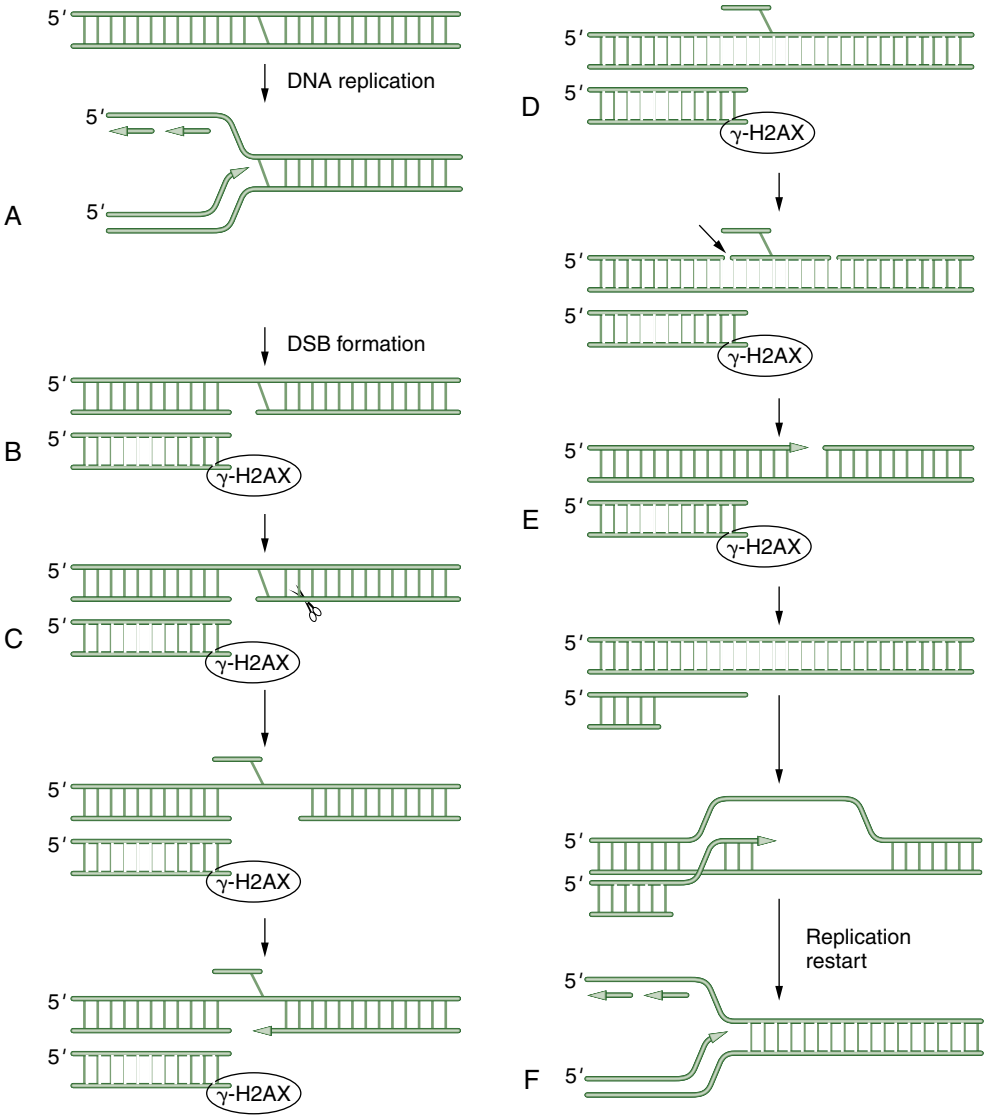


Figure 4-7 A schematic model of DNA crosslink repair DNA crosslink repair is believed to occur primarily during the S phase of the cell cycle. When a replication fork encounters an interstrand crosslink **(A)**, DNA replication arrests. Initially, a double-strand break is generated by an unknown endonuclease **(B)**. This DSB is next surrounded by phosphorylated histone 2AX. Next, another endonuclease (perhaps ERCC1/XPF) cleaves on the opposite side of the crosslink, allowing extrusion of the crosslinked bases from the double helix **(C)**. Next, a series of three DNA repair pathways act sequentially. Translesion synthesis (TLS) repair allows bypass of the damaged bases **(D)**. Then nucleotide excision repair (NER) excises the damaged oligonucleotide and allows gap filling **(E)**. Finally, end resection occurs, and the double-strand break can be repaired by homologous recombination (HR). The replication fork is regenerated in an error-free mechanism **(F)**.

and oxaliplatin) and mitomycin C, are especially cytotoxic to tumor cells, and their therapeutic index derives, at least in part, from the high proliferative rate of tumor cells versus normal cells.

The mechanism of DNA crosslink repair in human cells is poorly understood, and our understanding to date is derived more from the study of crosslink repair in prokaryotes and in the yeast *Saccharomyces cerevisiae*. As shown in Figure 4-7, crosslink repair in human cells probably requires multiple DNA repair pathways. According to this model, the ICL is only repaired during S phase progression. Initially, an advancing replication fork encounters an ICL. An unknown endonuclease cleaves the DNA, thus generating a double-strand break (DSB). Next, a second endonuclease is invoked to cleave the DNA after the DNA crosslink. Recent data suggest that this endonuclease may be made up of the ERCC1/XPF proteins.

Now that an endonucleolytic event has occurred on each side of the crosslink, the crosslinked single-strand fragment can be flipped out of the helix. This allows three of the normal DNA repair pathways to work sequentially. First, TLS allows bypass of the crosslink and replication and ligation of the upper double helix. Recent studies indicate that some variant polymerases, such as POL eta, are particularly important to the translesion synthesis across MMC adducts. Next, the NER pathway can excise a stretch of damaged DNA and allow gap filling of the excised oligonucleotide. Finally, HR repair can be used for the error-free, template-driven repair of the damage. The end result of this sequential use of three independent DNA repair pathways is to resume DNA replication and restart the replication fork.

Consistent with this model of crosslink repair, some repair-deficient cells are especially prone to the cytotoxic effects of DNA crosslinking drugs. For instance, cells that are

deficient in ERCC1/XPF generate the first double-strand break upstream of the DNA crosslink. However, these double-strand breaks, as measured indirectly by the presence of histone 2AX foci, persist in the repair-deficient cells, suggesting that ERCC1/XPF may work farther downstream in the pathway. Similarly, cells deficient in the FA pathway have persistent double-strand breaks after MMC exposure.[34] Thus, the presence or absence of the double-strand break intermediates is helpful in determining the level at which a repair process is disrupted and the sequence of repair events in the pathway.

DNA Repair and the DNA Damage Response

DNA repair is, in fact, only one class of a broader set of cellular responses referred to as the DNA damage response. DNA damage responses include the activation of cell cycle checkpoints, the activation of apoptosis, and the activation of DNA damage tolerance. This last mechanism allows a cell to "accept" DNA damage and continue DNA replication even in the setting of a heightened mutation frequency. The DNA damage response is therefore a highly coordinated set of signaling events. These responses require a DNA damage sensor (such as a sensor kinase, ATM, or ATR) and an effector kinase, as well as downstream protein machines dedicated to DNA repair, apoptosis, or checkpoint activities.[35]

The DNA Damage Response Is Mediated by Sensor and Effector Kinases

The DNA damage response can be activated by a wide range of environmental exposures or drug interactions. An important early player in the damage response is the molecular "sensor" of DNA damage. A local distortion in the DNA double helix, perhaps resulting from a DNA adduct or a thymine dimer, can activate a sensor kinase, such as ATM, ATR, or DNA-PK. These kinases are believed to autophosphorylate[36,37] and go on to phosphorylate a large number of substrates thereafter. The ATM kinase is the product of the ATM gene, the gene mutated in the cancer susceptibility disorder ataxia-telangiectasia.

Activated ATM and ATR proteins phosphorylate additional downstream "effector" kinases, such as the checkpoint kinases, Chk1 and Chk2. Activated Chk1 and Chk2 then go on to phosphorylate a wide array of protein targets involved in the machinery of DNA repair or DNA damage checkpoints.

One of the best-characterized DNA damage checkpoints is regulated by the ATM/Chk2/Cdc25A axis.[38,39] In response to IR, a double-strand break is generated, and this break activates ATM. ATM subsequently phosphorylates Chk2, which in turn phosphorylates the cell cycle activator cdc25A. Cdc25A phosphorylation leads to its rapid degradation and to cell-cycle arrest. This appears to be an important mechanism by which a cell can respond to DNA damage by arresting its cell cycle progression in S phase. By stopping S-phase entry, a cell allows itself the opportunity to slow down and to repair its DNA or, in the setting of severe damage, to undergo apoptosis. Importantly, a failure to activate this checkpoint response, as in ATM-deficient cells, results in S-phase progression even in the setting of DNA damage. Continuing to replicate DNA in the setting of DNA damage has dire consequences for the cells. The cell may have an elevated mutation rate or may complete DNA replication, only to experience a mitotic catastrophe at the end of the cell cycle.

Failure of ATM to activate the intra–S-phase checkpoint results in a characteristic cellular phenotype. When ATM-deficient cells are exposed to IR, they fail to arrest in S phase but instead continue to replicate their DNA and to incorporate tritiated thymidine in the postradiation period. This phenotype is known as radioresistant DNA synthesis (RDS), and it is the hallmark of a cell with a defect in the ATM/Chk2/cdc25A axis. An active area of DNA repair research is the identification of other CHK1 and CHK2 phosphorylated substrates.

Phosphorylated Effector Proteins Assemble in DNA Damage Foci

An important downstream event in the DNA damage response is the assembly of proteins in subnuclear foci.[40,41] These foci are often referred to as IRIFs (ionizing radiation inducible foci). Multiple ATM and ATR phosphorylated substrates, such as Chk1, BRCA1, and BARD1, assemble in foci following DNA damage. The assembly of these large protein complexes is mediated, at least in part, by the phosphorylated SQ or TQ sequences of the ATM/ATR substrates. Recent studies have indicated that these phosphorylated amino acid residues bind directly to phosphoamino acid receptors found on other adaptor proteins. For instance, phosphorylated BACH1 can bind directly to the BRCT domain (a phosphoserine receptor) of the BRCA1 protein.[42,43] The precise structure and function of these protein foci in eukaryote nuclei is not known. Clearly, the number of foci correlates with the number of unprocessed double-strand DNA breaks, and the foci are widely believed to be sites of double-strand break repair.

From immunofluorescence analysis, it is clear that multiple phosphorylated DNA-damage activated proteins colocalize in these foci. The foci have been helpful to researchers in the establishment of signaling pathways. For instance, pATM, pBRCA1, and pFANCD2 colocalize in IRIFs. Disruption of one upstream protein, say, by a germline or acquired mutation in the upstream signaling protein, ATM, results in loss of downstream proteins in the foci. The assembly of the foci therefore has become a useful tool in understanding the interrelationships of DNA response proteins.

A few DNA damage-response proteins deserve special attention here. Bonner and colleagues[40] have identified a variant histone protein, histone 2AX, that is rapidly phosphorylated by ATM after radiation damage. H2AX is an important early signaling protein in the DNA damage response. The phospho H2AX protein is incorporated in chromatin in vast stretches emanating from the site of the DNA DSB. An absence of histone 2AX, as in an H2AX knockout mouse model, results in chromosome instability and cancer predisposition,[44,45] apparently due to failure to mount the proper DNA damage response.

Another important DNA damage response protein is RAD51, which is phosphorylated by the Chk1 kinase during normal S-phase progression.[46] RAD51 is a single-strand DNA binding protein that plays a critical role in DNA repair by HR. Phosphorylated RAD51 also assembles in foci during normal S-phase progression. These "replication foci" are believed to be sites of DNA repair by HR between sister chromatids, which occurs during normal DNA replication.

A comprehensive analysis of proteins that are rapidly phosphorylated after DNA damage (and that form nuclear foci) has provided an important database for laboratories studying the DNA damage response. These phosphorylated proteins, and foci, provide a useful set of biomarkers for DNA repair activities. For instance, cells that are defective in the formation of DNA repair foci are themselves defective in DNA repair. The specific kind of focus that is absent correlates with the particular kind of DNA repair deficiency. For instance, cells deficient in RAD51 foci are defective in HR repair and are hypersensitive to IR. Cells defective in the assembly of polyADP ribose (PAR) foci are defective in the repair of single-strand breaks and may therefore have an underlying defect in BER. As such, tumor cells missing particular types of DNA repair foci may be more sensitive to certain kinds of chemotherapy or radiation.

Importantly, human cancers are often deficient in the DNA damage response. Germline mutations in DNA damage response genes, such as ATM, NBS1, FANCD2, BRCA1, and BRCA2, can result in an increased susceptibility to cancer. Individuals who inherit a single mutant allele of, say, BRCA1, have a high risk of developing a breast, ovarian, or prostate cancer during their lifetime. The tumor results from the inactivation of the second *BRCA1* allele, through deletion and loss of heterozygosity, thus resulting in a tumor with a specific DNA repair defect. BRCA(−/−) tumors therefore have genomic instability, but also have increased sensitivity to some DNA-damaging agents such as IR and DNA crosslinkers.

Study of the DNA damage response reveals that cells have highly regulated responses to different levels and types of DNA damage. Whereas some DNA repair pathways may be viewed as constitutive, housekeeping pathways, other pathways are highly controlled. Some DNA repair pathways, such as ATR and CHK1, are activated primarily at the site of the advancing replication fork, leading to the activation of HR repair.[47] Other DNA repair processes are activated in nondividing cells, such as in postmitotic neurons. For instance, NHEJ is hyperactive in nondividing cells and functions as the major mechanism of double-strand break repair in these cells.

The cellular context of the DNA repair pathway is also important. Germline or somatic disruption of a pathway may result in a strikingly different phenotype, depending on the cell and tissue of origin. For instance, gene line disruption of a DNA damage response, as in the inherited disease ataxia-telangiectasia, may lead to a characteristic constellation of clinical findings, including cerebellar degeneration and lymphoma predisposition. A somatic disruption of the same pathway (say, ATM CHk2-p53) may lead to a very different set of cancers, such as solid tumors of the bladder and ovary.[48,49]

Recent studies indicate that the DNA damage response provides an important "barrier" to the transformation of a normal cell to a malignant cell.[48,50] Specifically, early premalignant cells have heightened constitutive activation of the DNA damage response pathways, as exemplified by increased immunohistochemical staining with antibodies to activated ATM and to the activated checkpoint kinase CHK2. Interestingly, as cells progress from the premalignant state to the malignant state, they lose these DNA damage responses, perhaps through acquired disruptions of ATM or CHK2 activity. Because individuals with genetic diseases such as ataxia-telangiectasia already have a defect in the checkpoint response, they may be prone to earlier onset of cancers for this reason.

Inherited Chromosome Instability Syndromes as Models for DNA Repair Defects

Rare pediatric chromosome instability disorders, such as FA and XP, provide important insights into the function

of DNA repair pathways and their role in cancers in the general population. Children born with these syndromes generally have congenital abnormalities, cellular hypersensitivity to DNA-damaging agents, genomic instability, and an increased risk of specific cancers. Although these syndromes are rare, the DNA repair pathways disrupted by germline mutations in these individuals are often the same pathways disrupted by somatic mutation or epigenetic inactivation in cancers from the general population. For these sporadic cancers, a knowledge of which DNA repair mechanism is disrupted provides important clues to the behavior of the cancer or its drug sensitivity spectrum.

At least five of the major DNA repair pathways have corresponding inherited human diseases (Table 4-1). HR and TLS repair is defective in FA cells.[51] NER repair is defective in XP cells, Cockayne syndrome (CS) cells, and trichothiodystrophy cells.[52] MMR repair is defective in children with Turcot syndrome and in tumor cells derived from adult patients with HNPCC. TLS repair is defective in patients with the XP-V (xeroderma pigmentosum variant) disease. Most of these pediatric diseases exhibit autosomal recessive inheritance, such as XP, FA, and CS. Turcot syndrome has been reported to exhibit autosomal dominant or autosomal recessive inheritance depending on the particular mutation affecting mismatch repair. Inherited mutations in BER genes have not been observed in humans, suggesting that this pathway is essential for human development.

It is interesting that patients with inherited DNA repair syndromes, such as CS and FA, have congenital abnormalities. For instance, CS patients have developmental abnormalities of the skin and skeletal system. FA patients have skeletal, kidney, cardiac, and bone marrow defects. Consistent with these findings, the NER and FA pathways appear to play dual roles. For instance, the NER excision repair complex, TFIIH, plays an important transcriptional role during embryonic development. Germline dysfunction therefore leads to defects during embryonic organogenesis. The NER complex also plays a critical role in DNA repair in somatic cells after organism development. Similarly, the FA pathway appears to have a dual role in both development and DNA repair in somatic cells.

The systematic study of these rare diseases has led to (1) a better understanding of the genes and proteins involved in the six major DNA repair pathways, (2) how an inherited (or germline) defect in a DNA repair pathway can lead to genomic instability, cancer progression, and drug hypersensitivity, and (3) how an acquired (or somatic) defect in a DNA repair pathway can influence tumor progression and drug sensitivity of tumors in the general population. Although the specific details of these individual inherited diseases is beyond the scope of this review, an example of how a study of these rare diseases can lead to general insights to tumor biology can be appreciated from recent insights into the FA pathway.

Fanconi Anemia: A Specific Inherited DNA Repair Defect

FA is an autosomal recessive or X-linked recessive cancer susceptibility syndrome characterized by multiple congenital abnormalities, progressive bone marrow failure, and cellular hypersensitivity to DNA crosslinking agents, such as cisplatin and mitomycin C (MMC). FA patients are prone to developing acute myeloid leukemia as well as squamous cell carcinomas of the head and neck or gynecologic system.[53]

The study of FA cells has led to the elucidation of a DNA repair pathway for interstrand crosslinks. Clinically, this pathway is particularly important because many DNA crosslinking agents such as cisplatin or mitomycin C are used for cancer treatment. The FA defect results from biallelic mutation of any one of 15 known FA genes (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P). The proteins encoded by these FA genes cooperate in a common DNA repair pathway, referred to as the FA/BRCA pathway.[53,54] A central event in this pathway is the monoubiquitination of the FANCD2 protein, and this event is a useful biomarker for DNA repair activity (see later discussion). Disruption of this pathway results in the characteristic clinical and cellular phenotype of FA patients.

Patients with an Inherited Germline DNA Repair Deficiency Exhibit a Characteristic Tumor Spectrum

Patients with inherited DNA repair deficiency syndromes are prone to the development of specific tumors. FA patients, for example, are predisposed to acute myeloid leukemia and squamous cell carcinomas, primarily of the head and neck or gynecologic system. Patients with XP are prone to skin squamous cell carcinomas, primarily on body surfaces with more sunlight exposure. Patients with HNPCC and an inherited MMR deficiency are prone to colon cancer and ovarian cancer.

Tumors arising from somatic disruptions of DNA repair pathways may arise in other organ systems. A specific oncogenic lesion, such as the activation of an oncogene or the disruption of a tumor suppressor gene, may have a vastly different effect depending on the cellular context of the lesion. For instance, a germline mutation in the Rb gene may result in an embryonal tumor, such as a retinoblastoma or a pineoblastoma, but a somatic disruption of the Rb gene may lead to the development of a sarcoma.

Similarly, disruptions of a DNA repair pathway by a germline mechanism, rather than a somatic mechanism, may yield a very different spectrum and behavior. Examples are shown in Table 4-1. Somatic disruption of the FA pathway results in a wide range of tumor types, including tumors of the ovary, lung, and cervix.[51,55] Moreover, somatic disruptions result from methylation and silencing of an upstream FA gene (FANCF). Germline disruption of the same genes results from inherited mutations, such as missense mutations or nonsense mutations. Somatic disruption of the NER pathway plays a role in the development of testicular cancer and appears to account for the hypersensitivity of this tumor to the drug cisplatin. Paradoxically, somatic disruption of a DNA repair pathway can also result in chemotherapy resistance. Studies indicate that methylation and silencing of the MLH1 gene may account, at least in part, for the cisplatin resistance of some ovarian tumors.

Disruption of the other DNA repair pathways has been observed in sporadic human tumors, accounting, at least in part, for the specific drug and radiation sensitivity spectrum of these tumors and their clinical outcome. HR is disrupted in breast and ovarian cancer, NER is disrupted in testicular cancer, and MMR is disrupted in sporadic colon cancer. A few studies suggest that TLS may be disrupted in human cancers. Human cancer cells exhibit an elevation in spontaneous and damage-inducible point mutagenesis, compared to nonmalignant cells, suggesting an underlying TLS defect. Recent studies indicate that an elevation in the expression and activity of the error-prone polymerase Pol beta accounts for the increase in cisplatin resistance and mutagenesis of these cancers. Consistent with this hypothesis, inhibition of Pol beta in these cells results in resensitization to cisplatin.[56]

Somatic Disruption of DNA Repair Pathways by Methylation and Gene Silencing

One of the most common mechanisms of inactivation of DNA repair pathways in sporadic cancer is the epigenetic silencing of a critical gene through methylation of the promoter region. Increasing evidence shows that the FA/BRCA pathway is one of the DNA repair mechanisms that is targeted in sporadic cancers. *FANCF* methylation occurs in 24% of ovarian granulosa cell tumors, 30% of cervical cancer, 14% of squamous cell head and neck cancers, 6.7% of germ cell tumors of the testis, and 15% of non–small-cell lung cancers, where it correlates with a worse prognosis. An example of how methylation of a DNA repair gene can promote tumor progression is shown in Figure 4-8.

By regulating the activity of DNA repair pathways, cancer cells have a propensity to progress to a more malignant state. According to this model, early in the course of tumorigenesis, a premalignant cell may undergo a methylation and silencing of a DNA repair gene. In the case of the FA/BRCA pathway, the gene most commonly silenced by methylation is the *FANCF* gene on chromosome 11p15. Inactivation of *FANCF* results in a disruption of DNA repair and in genomic instability. The premalignant cell is therefore prone to multiple oncogenic events, such as the upregulation of a tyrosine kinase oncogene or the disruption of p53. A tumor with multiple somatic mutations eventually develops (see Figure 4-8), but this tumor still has a defective DNA repair pathway and is hypersensitive to genotoxic chemotherapy. After antitumor therapy, however, there is a selective pressure for tumor cells with an intact FA/BRCA

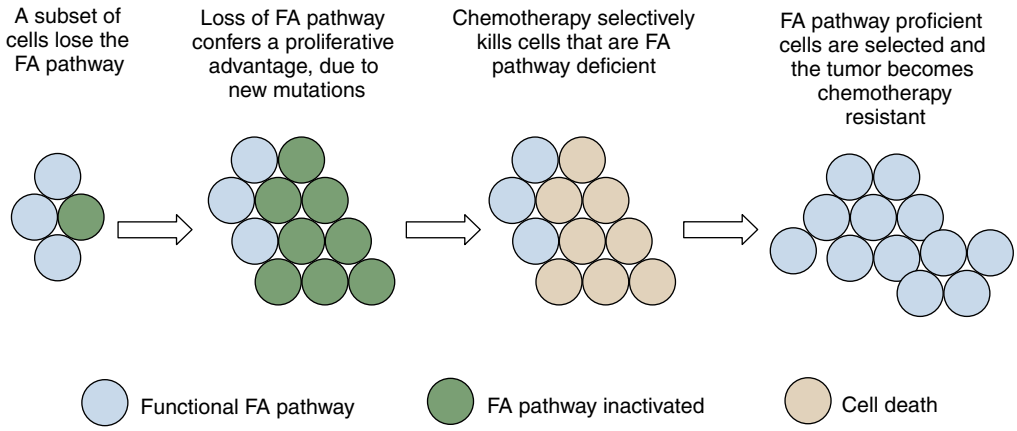


Figure 4-8 Tumor progression by serial inactivation and reactivation of DNA repair pathways According to this model, early in the course of carcinogenesis, a DNA repair pathway becomes inactivated. For instance, the *FANCF* gene may undergo biallelic methylation and silencing. This loss of the FA/BRCA pathway results in a state of chromosome instability, leading to secondary mutations (activation of K-Ras, inactivation of p53, for example). A tumor evolves, and the tumor is initially hypersensitive to cisplatin, as is often the case for ovarian epithelial cancer. Cisplatin causes rapid cytolysis of the tumor; however, rare tumor cells undergo a restoration of FANCF expression. Restoration may result either from an active demethylation of the *FANCF* gene or from positive selection of rare cells that experienced a stochastic demethylation event. Tumor cells regrow, and these cells are now cisplatin-resistant. In principle, an inhibitor of the FA/BRCA pathway can resensitize the tumor cells to cisplatin, as described in the text.

pathway. Tumor cells with a demethylated *FANCF* gene are selected, and a drug-resistant tumor emerges. By following this pattern, tumors can silence and reactivate DNA repair pathways, leading to drug resistance and tumor progression.

The converse scenario may occur for the MMR pathway. MMR-proficient cells are hypersensitive to the DNA crosslinking drug cisplatin. In this case, it is believed that the active MMR pathway generates a cisplatin-inducible lesion that is tumoricidal. Inactivation of MMR by methylation of the MSH2 gene therefore provides the tumor with a mechanism for achieving cisplatin resistance. Based on these examples, it appears that understanding the methylation state of various DNA repair genes may allow the prediction of drug responsiveness of some tumors.

Epigenetic silencing of *BRCA1* through methylation occurs in 13% of breast cancers, 23% of advanced ovarian cancers, 6% of cervical cancers, and 4% of non–small-cell lung cancers. Epigenetic disruption of the FA pathway may also be important in the development of sporadic acute myeloid leukemia (AML), where absent or reduced expression of the FA proteins FANCA, FANCC, FANCF, and FANCG has been reported. Loss of BRCA2 mRNA and protein expression has been reported in 13% of ovarian adenocarcinomas; in contrast to the other FA genes described earlier, this loss does not result from promoter methylation.

Prognostic and Predictive DNA Repair Biomarkers in Cancer Treatment

Both hereditary cancer syndromes and sporadic cancers can arise from abnormalities in DNA repair pathways. This may be clinically important because these tumors are expected to be hypersensitive to DNA-damaging therapeutic agents or strategies that inhibit alternative DNA repair pathways. In the case of sporadic cancers, the patient's normal cells, such as those in the bone marrow, possess a functional DNA repair pathway and are predicted to be resistant to these targeted treatments. Assessment of the status of the FA pathway or other DNA repair pathways requires the use of diagnostic biomarkers.

Selection of Biomarkers of DNA Repair Pathways

Biomarkers of DNA repair pathways can be divided into two major groups: functional biomarkers that characterize the activity of a pathway following damage and expression biomarkers that measure the availability of pathway components before damage.

Functional DNA Repair Biomarkers

These are biomarkers that indicate an intact DNA repair pathway. These biomarkers have the advantage of giving a functional measure of a particular pathway and will detect repair defects due to epigenetic events or gene mutations. Moreover, they give a global measurement of a particular pathway's function without the need to know the identities of all the components. They could also be used to differentiate between insignificant single-nucleotide polymorphisms (SNPs) and functionally important point mutations in DNA repair pathway genes. Functional biomarkers can be applied to serial tumor samples from the same patient, at diagnosis and at the time of relapse. In this way, one can determine whether the tumor remains drug sensitive or has restored its DNA repair mechanisms. However, these markers rely on tumor tissue having been exposed to some form of DNA damage in vivo or in vitro before the assay. Functional biomarkers of DNA repair pathways include the monoubiquitination of the FANCD2 protein (a biomarker for HR repair) and the phosphorylation of DNA-PK (a biomarker of a functional NHEJ pathway). Abnormal DNA-damage–induced nuclear foci may identify disruption of the downstream events in the pathway, such as that observed in BRCA1- or BRCA2-deficient cells.

DNA Repair Biomarkers of Gene/Protein Expression

These biomarkers indicate the preexisting function of a DNA damage pathway before damage. Examples are real-time polymerase chain reaction (rt-PCR) or immunohistochemistry to test for epigenetic silencing of critical DNA repair genes. Some studies have used a microarray approach to look for genetic expression profiles indicative of abnormal DNA repair gene function. Because some DNA repair genes, such as MLH1 and MSH2, are inactivated by methylation, measurement of gene methylation via the methylation-PCR assay can also be applied as a biomarker assay. These approaches have the advantage of not requiring prior DNA damage and can be performed on fixed specimens. However, they provide only an indirect measurement of the functional capabilities of a DNA repair pathway. In addition, mutant genes can express normal levels of mRNA, and mutant protein and would not be detected by this method.

Clinical Application of DNA Repair Biomarkers

DNA Repair Biomarkers as Predictors of Response to Conventional Therapy

Loss or increased activity of particular DNA repair pathways may influence the response to DNA-damaging therapeutic strategies. For instance, a failure of a pathway

involved in the repair of DNA crosslinks such as HR would be predicted to sensitize a tumor to DNA crosslinking agents such as alkylating chemotherapeutic drugs. Indeed, BRCA1 expression levels as measured by rt-PCR have been used as a biomarker of survival following cisplatin-based chemotherapy for non–small-cell lung cancer. Methylation-specific PCR, which indicates loss of gene expression through promoter methylation, has been used to correlate loss of BRCA1 function with cisplatin sensitivity in ovarian cancer. Loss of *BRCA2/FANCD1* function through mutation in breast or ovarian cancer has also been reported to correlate with a high response to DNA-damaging chemotherapeutic agents. Absence of FANCD2 monoubiquitination may be a biomarker for loss of function of upstream FA pathway components and could be expected to predict sensitivity to DNA crosslinkers such as cisplatin or cyclophosphamide.

A recent example of the use of DNA repair biomarker in clinical medicine is the evaluation of ERCC1 protein expression levels in lung cancer. The NER pathway is important for the correction of UV-light–induced thymine dimers and for the excision of small single-base adducts. In addition, two of the proteins involved in NER, ERCC1 and XP-F, appear to have special relevance to the repair of DNA interstrand crosslinks. Primary cells derived from XP patients with germline mutations in ERCC1 or XPF are hypersensitive to UV and to DNA crosslinking agents.[57] Recent studies also suggest that the protein level of ERCC1 in cell lines correlates with the level of functional DNA crosslink repair in the cell.

Several groups have performed retrospective analyses of non–small-cell lung cancer patients who had been treated with adjuvant chemotherapy, including cisplatin. The banked primary tumor samples, which were stored in paraffin blocks, were evaluated for the level of ERCC1 protein by immunohistochemistry (IHC). Interestingly, the patients with tumors exhibiting low ERCC1 levels were more sensitive to cisplatin, based on their longer average time to relapse after cisplatin, compared to patients whose tumors had high levels of ERCC1. Together these studies indicate that ERCC1 protein expression may be a useful predictive biomarker for assessing tumor response to cisplatin.

DNA Repair Biomarkers to Guide Chemo- and Radiosensitization

Resistance to DNA-damaging chemotherapy or radiotherapy may be due to enhanced repair of DNA lesions. Therefore, a possible therapeutic strategy is to use drugs that specifically inhibit DNA repair pathways. Theoretically, this strategy may be limited because the drug may also increase the toxicity of therapeutic DNA damage in normal tissue. A therapeutic index can be achieved, in principle, by (1) selective uptake of the DNA damage sensitizers by the tumor cell, versus the normal cell, or (2) delivering one of the modalities (such as the radiation) directly to the tumor.

An understanding of the precise molecular mechanisms of new classes of sensitizing agents has important implications. First, if an agent functions by inhibiting a specific DNA repair pathway, then active derivatives of this agent should function similarly. DNA repair pathway inhibition provides an important biomarker for determining the proper dosing of the drug. Second, the chemosensitizer would be predicted to be more efficacious when used in combination with specific classes of DNA damage drugs.

DNA Repair Biomarkers as Predictors of Response to Targeted Monotherapy

Another important application for biomarkers of DNA repair pathway integrity is the potential to develop nontoxic monotherapy for tumors with specific DNA repair defects. The upregulated DNA repair pathway is the "Achilles heel" of the cancer. In principle, a nontoxic inhibitor of this second pathway, delivered as a monotherapy, may selectively kill the cancer cell. A normal cell, in comparison, may be able to tolerate the loss of this second pathway because other pathways are functioning and there is more redundancy in its DNA repair capacity.

This principle has recently been demonstrated by the use of PARP inhibitors in BRCA1- and BRCA2-deficient cells.[11,12] As discussed earlier, under normal physiological conditions DNA is being damaged continuously. The result of these stresses is the development of damaged bases or regions of single-strand DNA breaks that are repaired through the BER pathway. Part of the BER pathway requires PARP, a DNA-binding zinc finger protein that catalyzes the transfer of ADP-ribose residues from NAD^+ to itself and different chromatin constituents, forming branched ADP-ribose polymers. Initially it was observed that PARP-deficient (and therefore BER-deficient) mice develop normally but have high levels of sister chromatid exchange, a feature of HR. This observation suggested that HR could compensate for a loss of PARP-dependent BER. Consequently it was demonstrated in preclinical models that BRCA1- and BRCA2-deficient human and murine cells were sensitive to PARP-inhibiting drugs, whereas cells expressing normal levels of BRCA1 or BRCA2 were unaffected. PARP1 inhibitors are well tolerated in preclinical murine models and, in addition to being a potential treatment for BRCA1 and BRCA2 mutant tumors, may also represent an attractive strategy for chemoprevention of malignancies in mutation carriers. Clearly a biomarker that indicates a failure of BRCA1 or BRCA2 function in tumor cells may allow the application of PARP inhibitors to a wider spectrum of sporadic human malignancies.

The Development of New DNA Repair Biomarkers

Few biomarkers exist at present for evaluating the integrity of the other DNA repair pathways. Several studies have attempted to assay these pathways, using expression biomarkers (i.e., testing the expression levels of known DNA repair proteins in the pathways.) Better functional biomarkers are needed. Recent studies have indicated that posttranslational modifications of DNA repair proteins in these pathways are also required for pathway activity. For instance, polyubiquitination of XP-C is required for functional NER,[24] and sumoylation of thymine-DNA glycosylase[22] is required for function of BER. The development of antibodies specific for these activated states and the testing of these biomarkers may allow the rapid assessment of drug sensitivity and acquired resistance in clinical samples.

DNA Repair Inhibitors as a New Area for Anticancer Drug Development

As shown in Figure 4-9, normal human cells may have six functional DNA repair pathways, whereas a tumor cell may have disruptions of one pathway. In the tumor, disruption of one pathway, such as HR repair, results in genomic instability and hyperdependence on a second pathway, such as BER. Because of this hyperdependent state, the tumor cell may be hypersensitive to an inhibitor of BER, such as a PARP1 inhibitor.

In this case, the tumor may respond to the PARP1 inhibitor as a single agent (monotherapy).[58-60] DNA damage, activated by the hyperproliferative state of the tumor cell, may be sufficient to kill the tumor cell. Alternatively, the PARP1 inhibitor may have a greater tumoricidal effect when it is used in combination with another cytotoxic agent, such as IR or an alkylating agent (TMZ).

Based on the early success with PARP1 inhibitor therapy, there is increasing interest in the identification of inhibitors of other DNA repair pathways.[61] For instance, an inhibitor of the sensor kinase ATM has been shown to have potent tumoricidal effects.[62] Also, inhibitors of the Chk1 kinase UCNO1 have been used in clinical trials.[35,63,64] Investigators have also begun to screen for inhibitors of HR repair that may potentially sensitize tumor cells to IR or to crosslinker damage.[65,66] Although DNA repair inhibitors may sensitize a tumor to the cytotoxic activity of conventional IR or chemotherapy, they may also enhance the toxicity of these therapies to normal human cells.

Importance of DNA Repair to Clinical Oncology: Other Specific Examples

BRCA1 and BRCA2

The importance of DNA repair to the pathogenesis and treatment of cancer is exemplified by studies of the breast cancer susceptibility gene BRCA1. Approximately 10% of women who develop breast cancer in their lifetime have a strong family history of (inherited) breast cancer. Of these women, approximately half are heterozygous carriers for mutations in either the *BRCA1* or *BRCA2* gene.

The *BRCA1* gene was originally mapped to human chromosome 17,[67] and it was subsequently cloned by position.[68] Strong evidence emerged that *BRCA1* is a tumor suppressor gene, since breast carriers have loss of heterozygosity at the *BRCA1* locus.[69] *BRCA1*-deficient breast tumor cells are hypersensitive to IR and to DNA crosslinking agents,

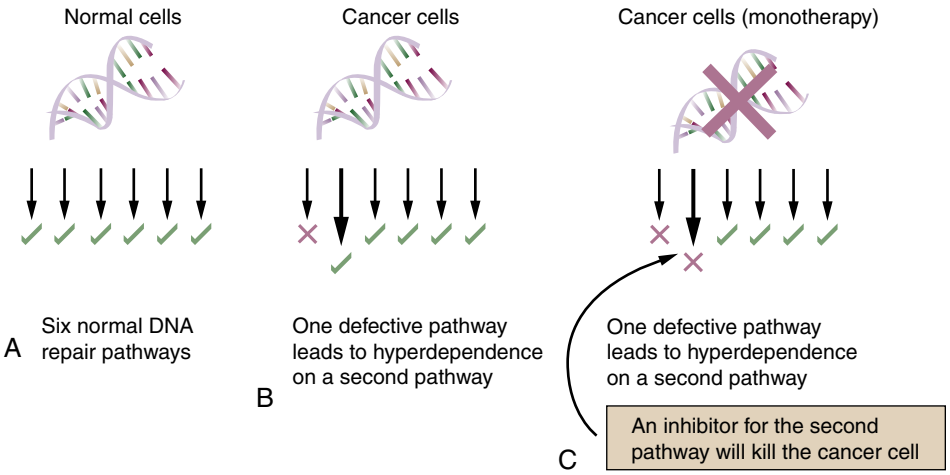


Figure 4-9 Principle of DNA inhibitor monotherapy (A). Normal human cells have six DNA repair pathways. **(B)** Tumor cells, in contrast, have disrupted one DNA repair pathway through somatic mutation, loss of heterozygosity (LOH), or epigenetic silencing of a DNA repair gene in that pathway. The tumor cell has genomic instability and has partially compensated for its DNA repair defect by upregulating a second pathway. **(C)** The tumor is hyperdependent on this second pathway, and a specific inhibitor kills the tumor cells but has little effect on the normal cells. An example of this monotherapy approach has been described for PARP inhibitors.[11,12]

suggesting that *BRCA1* may function in the regulation of HR repair.

Studies with the BRCA1 protein indicate that, during the DNA damage response following cellular exposure to a genotoxic stress, BRCA1 is phosphorylated and accumulates in subnuclear foci that colocalize with BRCA2 and RAD51 proteins.[70,71] These foci are required for competent DNA repair. The precise role of BRCA1 in DNA repair is unknown. Because BRCA1 is itself an E3 ubiquitin ligase,[72] it may function by ubiquitinating other DNA repair proteins and regulating DNA repair indirectly. Recent studies have identified key ubiquitinated substrates of BRCA1, including the protein CTIP.[73]

Because BRCA1 (and BRCA2) tumors are deficient in HR repair, this genotype may be useful in the selection of chemotherapy, as described earlier for these tumors. Recent studies indicate that BRCA1-deficient tumors are hyperdependent on BER and have elevated PARP1 activity.[11,12] Accordingly, BRCA1- and BRCA2-deficient tumors appear to be hypersensitive to PARP1 inhibitors.

Defects in DNA Repair Pathways Can Account for the Elevated Mutation Rate of Cancer

Cancer cells have an increased mutation rate compared to normal cells, and this phenotype has important clinical consequences. The increased mutation frequency can lead to point mutation and inactivation of tumor suppressor genes or to increased tumor cell resistance to chemotherapy. The increased mutation rate may also account for the increased spontaneous cell death observed in solid tumor samples (i.e., some of the mutations may be lethal to individual tumor cells), but may also enhance the outgrowth of a more malignant clone.

This increased mutation rate results in large part through the disruption of DNA repair pathways. The MMR pathway normally functions to improve the fidelity of DNA replication by quickly identifying and excising mismatched bases generated by faulty DNA replication. Loss of the MMR pathway by germline or somatic mutation can lead to a "mutator" phenotype. This phenotype can be readily detected by microsatellite instability in the genome of the cancer cell.

This increase in mutation rate can also be accounted for by an increase in error-prone DNA repair mechanisms.[74] In the setting of elevated translesion synthesis, some error-prone polymerases, such as Rev3, may increase the frequency of point mutations in the genome of the human cancer. Also, an elevation in the error-prone NHEJ pathway may account for the elevated complex mutations (insertions and deletions) observed in some cancers. Many human tumors have recently been found to express abnormal levels of polymerase β,[75,76] which may also contribute to their increased mutation frequency.

Multiple Mechanisms of Cisplatin Resistance

Recent studies indicate that the status of DNA repair pathways in human tumors may be highly predictive of cisplatin sensitivity. As mentioned previously, defects in the NER pathway may account for cisplatin sensitivity of some testicular and non–small-cell lung cancers.

Defects in HR repair may account for the cisplatin sensitivity of ovarian and head and neck carcinomas. Other cellular mechanisms may also account for the intrinsic cisplatin resistance of many human tumors. Cisplatin-mediated tumoricidal activity can be affected by (1) the expression of cell surface P-glycoprotein, an efflux mechanism for removing cisplatin, and (2) the relative antiapoptotic state of the tumor cell, based at least in part of the level of BCL-2 and BCL-X expression. Primary cisplatin resistance may therefore rely on the systematic assay of many of these mechanisms in a given tumor cell.[77]

DNA Repair Gene Polymorphisms as Predictors of Chemotherapy Responsiveness

As described earlier, disruption of the NER pathway appears to account, at least in part, for the cisplatin hypersensitivity of testicular cancers and of some ERCC1-deficient non–small-cell lung cancers.[17] The disruption of the NER pathway may result from definitive mutations (i.e., frame-shift or nonsense mutations) in NER genes or from epigenetic changes, such as methylation and silencing of NER genes. In some cases, the disruption of the NER pathway may be partial, and it may result from DNA repair gene polymorphisms carried in the germline of the cancer patient.

In principle, DNA repair polymorphisms may result in a subtler DNA repair defect. Such a defect may increase the risk that an individual develops a cancer or may increase the likelihood that the resulting tumor is sensitive to a specific genotoxic agent. Based on this idea, investigators have screened large tumor sets for the enrichment of particular SNPs in DNA repair genes. Common SNPs are known for the NER genes XPD, ERCC1, and XRCC1. SNPs in multiple NER genes appear to account, at least in part, for the cisplatin hypersensitivity of some squamous cell carcinomas and lung cancers. Whether these SNPs will serve as

predictive biomarkers for chemotherapy or radiation sensitivity remains unproven.

Conclusion

Genomic instability is characteristic of most human malignancies, and this phenotype can arise from acquired defects in any one of six DNA repair pathways. These pathways are MMR, BER, NHEJ, NER, HR, and TLS. The germline disruption of these pathways accounts for the pathogenesis of several inherited DNA repair disorders including FA, XP, and HNPCC. The somatic disruption of these pathways can account for the genomic instability and drug sensitivity of many tumor types. The six pathways differ significantly in their ability to repair modified DNA bases and DNA crosslinks. Different cell types and tumor cell types have differential dependence on these pathways for growth and survival.

In the future, the development of biomarkers for the function of other DNA repair pathways may allow the better targeting of conventional agents or the use of monotherapies designed to inhibit specific repair pathways. The biomarkers can also be used as screening tools to find inhibitors of DNA repair that function as chemosensitizers. We predict that these approaches should reduce the toxicity of existing cancer treatments by eliminating the use of noneffective agents and by directing the development of novel treatment strategies.

Understanding the status of DNA repair pathways in tumor cells will have considerable use in clinical oncology. If a tumor is defective in one pathway (say, NHEJ), it may be directly sensitive to IR, a modality that generates double-strand breaks in DNA. If a tumor is defective in another pathway (say, HR) it may be hyperdependent on a second pathway (say, BER) for its survival. Accordingly, a drug, such as a PARP1 inhibitor, that targets the BER pathway may be selectively toxic to these tumors. Recent studies indicate new synthetic lethal relationships among DNA repair pathways. MMR-deficient tumors are hypersensitive to ATM inhibition.[78] A complete understanding of the synthetic lethal relationships of all six of the major DNA repair pathways is an important future goal of DNA repair research in cancer.[66]

Key References

5. Surralles J, Jackson SP, Jasin M, Kastan MB, West SC, Joenje H. Molecular cross-talk among chromosome fragility syndromes. *Genes Dev*. 2004;18(12):1359-1370.
6. Modrich P. Mechanisms in eukaryotic mismatch repair. *J Biol Chem*. 2006;281(41):30305-30309.
7. Friedberg EC, Lehmann AR, Fuchs RP. Trading places: how do DNA polymerases switch during translesion DNA synthesis? *Mol Cell*. 2005;18(5):499-505.
8. Boulton SJ. Cellular functions of the BRCA tumour-suppressor proteins. *Biochem Soc Trans*. 2006;34(Pt 5):633-645.
9. Nojima K, Hochegger H, Saberi A, et al. Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. *Cancer Res*. 2005;65(24):11704-11711.
10. Friedberg EC. DNA damage and repair. *Nature*. 2003;421(6921):436-440.
11. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434(7035):913-917.
12. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434(7035):917-921.
14. Nitta M, Kozono D, Kennedy R, et al. Targeting EGFR induced oxidative stress by PARP1 inhibition in glioblastoma therapy. *PLoS One*. 2010;5(5):e10767.
15. Martin SA, McCabe N, Mullarkey M, et al. DNA polymerases as potential therapeutic targets for cancers deficient in the DNA mismatch repair proteins MSH2 or MLH1. *Cancer Cell*. 2010;17(3):235-248.
17. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*. 2006;355(10):983-991.
18. Lieber MR, Ma Y, Pannicke U, Schwarz K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol*. 2003;4(9):712-720.
19. Fischhaber PL, Friedberg EC. How are specialized (low-fidelity) eukaryotic polymerases selected and switched with high-fidelity polymerases during translesion DNA synthesis? *DNA Repair (Amst)*. 2005;4(2):279-283.
20. McCulloch SD, Kunkel TA. Measuring the fidelity of translesion DNA synthesis. *Methods Enzymol*. 2006;408:341-355.
21. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352(10):997-1003.
22. Steinacher R, Schar P. Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol*. 2005;15(7):616-623.
23. Ulrich HD. SUMO modification: wrestling with protein conformation. *Curr Biol*. 2005;15(7):R257-R259.
24. Sugasawa K. UV-induced ubiquitylation of XPC complex, the UV-DDB-ubiquitin ligase complex, and DNA repair. *J Mol Histol*. 2006;37(5-7):189-202.
25. Huang TT, D'Andrea AD. Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol*. 2006;7(5):323-334.
26. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. Gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell*. 2005;20(5):801-809.
27. Nijman SM, Huang TT, Dirac AM, et al. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol Cell*. 2005;17(3):331-339.
28. Huang TT, Nijman SM, Mirchandani KD, et al. Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nat Cell Biol*. 2006;8(4):339-347.
29. Yang K, Moldovan GL, Vinciguerra P, Murai J, Takeda S, D'Andrea AD. Regulation of the Fanconi anemia pathway by a SUMO-like delivery network. *Genes Dev*. 2011;25(17):1847-1858.
30. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011;331(6024):1553-1558.
31. Smogorzewska A, Matsuoka S, Vinciguerra P, et al. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell*. 2007;129(2):289-301.
32. Paulsen RD, Soni DV, Wollman R, et al. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell*. 2009;35(2):228-239.
33. O'Connell BC, Adamson B, Lydeard JR, et al. A genome-wide camptothecin sensitivity screen identifies a mammalian MMS22L-NFKBIL2 complex required for genomic stability. *Mol Cell*. 2010;40(4):645-657.
34. Rothfuss A, Grompe M. Repair kinetics of genomic interstrand DNA cross-links: evidence for DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway. *Mol Cell Biol*. 2004;24(1):123-134.

35. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004;432(7015):316-323.
36. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. 2003;421(6922):499-506.
37. Bakkenist CJ, Kastan MB. Initiating cellular stress responses. *Cell*. 2004;118(1):9-17.
39. Sorensen CS, Syljuasen RG, Falck J, et al. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. *Cancer Cell*. 2003;3(3):247-258.
40. Nakamura A, Sedelnikova OA, Redon C, et al. Techniques for gamma-H2AX detection. *Methods Enzymol*. 2006;409:236-250.
41. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nat Cell Biol*. 2004;6(2):168-170.
42. Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science*. 2003;302(5645):636-639.
43. Yu X, Chini CC, He M, Mer G, Chen J. The BRCT domain is a phosphoprotein binding domain. *Science*. 2003;302(5645):639-642.
45. Franco S, Gostissa M, Zha S, et al. H2AX prevents DNA breaks from progressing to chromosome breaks and translocations. *Mol Cell*. 2006;21(2):201-214.
46. Sorensen CS, Hansen LT, Dziegielewski J, et al. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol*. 2005;7(2):195-201.
47. Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev*. 2004;18(16):1958-1963.
48. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anticancer barrier in early human tumorigenesis. *Nature*. 2005;434(7035):864-870.
49. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*. 2006;444(7119):633-637.
50. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*. 2005;434(7035):907-913.
51. Kennedy RD, D'Andrea AD. DNA repair pathways in clinical practice: lessons from pediatric cancer susceptibility syndromes. *J Clin Oncol*. 2006;24(23):3799-3808.
52. Giglia-Mari G, Coin F, Ranish JA, et al. A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat Genet*. 2004;36(7):714-719.
53. Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev*. 2005;19(24):2925-2940.
54. Kee Y, D'Andrea AD. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev*. 2010;24(16):1680-1694.
55. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. *Blood*. 2006;107(11):4223-4233.
56. Boudsocq F, Benaim P, Canitrot Y, et al. Modulation of cellular response to cisplatin by a novel inhibitor of DNA polymerase beta. *Mol Pharmacol*. 2005;67(5):1485-1492.
57. Niedernhofer LJ, Lalai AS, Hoeijmakers JH. Fanconi anemia (cross)linked to DNA repair. *Cell*. 2005;123(7):1191-1198.
58. Lord CJ, Garrett MD, Ashworth A. Targeting the double-strand DNA break repair pathway as a therapeutic strategy. *Clin Cancer Res*. 2006;12(15):4463-4468.
59. McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res*. 2006;66(16):8109-8115.
60. Tutt AN, Lord CJ, McCabe N, et al. Exploiting the DNA repair defect in BRCA mutant cells in the design of new therapeutic strategies for cancer. *Cold Spring Harb Symp Quant Biol*. 2005;70:139-148.
61. Sanchez-Perez I. DNA repair inhibitors in cancer treatment. *Clin Transl Oncol*. 2006;8(9):642-646.
62. Hickson I, Zhao Y, Richardson CJ, et al. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res*. 2004;64(24):9152-9159.
63. Syljuasen RG, Sorensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation. *Cancer Res*. 2004;64(24):9035-9040.
64. Ma CX, Cai S, Li S, et al. Targeting Chk1 in p53-deficient triple-negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. *J Clin Invest*. 2012;122(4):1541-1552.
65. Chirnomas D, Taniguchi T, de la Vega M, et al. Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. *Mol Cancer Ther*. 2006;5(4):952-961.
66. Jenkins C, Kan J, Hoatlin ME. Targeting the Fanconi anemia pathway to identify tailored anticancer therapeutics. *Anemia*. 2012;2012:481-483.
69. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*. 2006;25(43):5864-5874.
73. Yu X, Fu S, Lai M, Baer R, Chen J. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev*. 2006;20(13):1721-1726.
74. Burr KL, Velasco-Miguel S, Duvvuri VS, McDaniel LD, Friedberg EC, Dubrova YE. Elevated mutation rates in the germline of Polkappa mutant male mice. *DNA Repair (Amst)*. 2006;5(7):860-862.
75. Sweasy JB, Lang T, DiMaio D. Is base excision repair a tumor suppressor mechanism? *Cell Cycle*. 2006;5(3):250-259.
76. Sweasy JB, Lauper JM, Eckert KA. DNA polymerases and human diseases. *Radiat Res*. 2006;166(5):693-714.
77. Bagby GC, Olson SB. Cisplatin and the sensitive cell. *Nat Med*. 2003;9(5):513-514.
78. Kennedy RD, Chen CC, Stuckert P, et al. Fanconi anemia pathway-deficient tumor cells are hypersensitive to inhibition of ataxia telangiectasia mutated. *J Clin Invest*. 2007;117(5):1440-1449.

This page intentionally left blank

References

1. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature*. 1998;396(6712):643-649.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.
3. Cleaver JE. Defective repair replication of DNA in xeroderma pigmentosum. *Nature*. 1968;218(5142):652-656.
4. Cleaver JE. Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective. *Proc Natl Acad Sci U S A*. 1969;63(2):428-435.
5. Surralles J, Jackson SP, Jasin M, Kastan MB, West SC, Joenje H. Molecular cross-talk among chromosome fragility syndromes. *Genes Dev*. 2004;18(12):1359-1370.
6. Modrich P. Mechanisms in eukaryotic mismatch repair. *J Biol Chem*. 2006;281(41):30305-30309.
7. Friedberg EC, Lehmann AR, Fuchs RP. Trading places: how do DNA polymerases switch during translesion DNA synthesis? *Mol Cell*. 2005;18(5):499-505.
8. Boulton SJ. Cellular functions of the BRCA tumour-suppressor proteins. *Biochem Soc Trans*. 2006;34(Pt 5):633-645.
9. Nojima K, Hochegger H, Saberi A, et al. Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. *Cancer Res*. 2005;65(24):11704-11711.
10. Friedberg EC. DNA damage and repair. *Nature*. 2003;421(6921):436-440.
11. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*. 2005;434(7035):913-917.
12. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434(7035):917-921.
13. Wilson SH, Sobol RW, Beard WA, Horton JK, Prasad R, Vande Berg BJ. DNA polymerase beta and mammalian base excision repair. *Cold Spring Harb Symp Quant Biol*. 2000;65:143-155.
14. Nitta M, Kozono D, Kennedy R, et al. Targeting EGFR induced oxidative stress by PARP1 inhibition in glioblastoma therapy. *PLoS One*. 2010;5(5):e10767.
15. Martin SA, McCabe N, Mullarkey M, et al. DNA polymerases as potential therapeutic targets for cancers deficient in the DNA mismatch repair proteins MSH2 or MLH1. *Cancer Cell*. 2010;17(3):235-248.
16. Friedberg EC. How nucleotide excision repair protects against cancer. *Nat Rev Cancer*. 2001;1(1):22-33.
17. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*. 2006;355(10):983-991.
18. Lieber MR, Ma Y, Pannicke U, Schwarz K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol*. 2003;4(9):712-720.
19. Fischhaber PL, Friedberg EC. How are specialized (low-fidelity) eukaryotic polymerases selected and switched with high-fidelity polymerases during translesion DNA synthesis? *DNA Repair (Amst)*. 2005;4(2):279-283.
20. McCulloch SD, Kunkel TA. Measuring the fidelity of translesion DNA synthesis. *Methods Enzymol*. 2006;408:341-355.
21. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352(10):997-1003.
22. Steinacher R, Schar P. Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol*. 2005;15(7):616-623.
23. Ulrich HD. SUMO modification: wrestling with protein conformation. *Curr Biol*. 2005;15(7):R257-R259.
24. Sugasawa K. UV-induced ubiquitylation of XPC complex, the UV-DDB-ubiquitin ligase complex, and DNA repair. *J Mol Histol*. 2006;37(5-7):189-202.
25. Huang TT, D'Andrea AD. Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol*. 2006;7(5):323-334.
26. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. Gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell*. 2005;20(5):801-809.
27. Nijman SM, Huang TT, Dirac AM, et al. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol Cell*. 2005;17(3):331-339.
28. Huang TT, Nijman SM, Mirchandani KD, et al. Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nat Cell Biol*. 2006;8(4):339-347.
29. Yang K, Moldovan GL, Vinciguerra P, Murai J, Takeda S, D'Andrea AD. Regulation of the Fanconi anemia pathway by a SUMO-like delivery network. *Genes Dev*. 2011;25(17):1847-1858.
30. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011;331(6024):1553-1558.
31. Smogorzewska A, Matsuoka S, Vinciguerra P, et al. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell*. 2007;129(2):289-301.
32. Paulsen RD, Soni DV, Wollman R, et al. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol Cell*. 2009;35(2):228-239.
33. O'Connell BC, Adamson B, Lydeard JR, et al. A genome-wide camptothecin sensitivity screen identifies a mammalian MMS22L-NFKBIL2 complex required for genomic stability. *Mol Cell*. 2010;40(4):645-657.
34. Rothfuss A, Grompe M. Repair kinetics of genomic interstrand DNA cross-links: evidence for DNA double-strand break-dependent activation of the Fanconi anemia/BRCA pathway. *Mol Cell Biol*. 2004;24(1):123-134.
35. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004;432(7015):316-323.
36. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. 2003;421(6922):499-506.
37. Bakkenist CJ, Kastan MB. Initiating cellular stress responses. *Cell*. 2004;118(1):9-17.
38. Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature*. 2001;410(6830):842-847.
39. Sorensen CS, Syljuasen RG, Falck J, et al. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. *Cancer Cell*. 2003;3(3):247-258.
40. Nakamura A, Sedelnikova OA, Redon C, et al. Techniques for gamma-H2AX detection. *Methods Enzymol*. 2006;409:236-250.
41. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nat Cell Biol*. 2004;6(2):168-170.
42. Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science*. 2003;302(5645):636-639.
43. Yu X, Chini CC, He M, Mer G, Chen J. The BRCT domain is a phosphoprotein binding domain. *Science*. 2003;302(5645):639-642.
44. Celeste A, Petersen S, Romanienko PJ, et al. Genomic instability in mice lacking histone H2AX. *Science*. 2002;296(5569):922-927.
45. Franco S, Gostissa M, Zha S, et al. H2AX prevents DNA breaks from progressing to chromosome breaks and translocations. *Mol Cell*. 2006;21(2):201-214.
46. Sorensen CS, Hansen LT, Dziegielewski J, et al. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nat Cell Biol*. 2005;7(2):195-201.
47. Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev*. 2004;18(16):1958-1963.
48. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 2005;434(7035):864-870.
49. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*. 2006;444(7119):633-637.
50. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*. 2005;434(7035):907-913.
51. Kennedy RD, D'Andrea AD. DNA repair pathways in clinical practice: lessons from pediatric cancer susceptibility syndromes. *J Clin Oncol*. 2006;24(23):3799-3808.
52. Giglia-Mari G, Coin F, Ranish JA, et al. A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat Genet*. 2004;36(7):714-719.
53. Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev*. 2005;19(24):2925-2940.
54. Kee Y, D'Andrea AD. Expanded roles of the Fanconi anemia pathway in preserving genomic stability. *Genes Dev*. 2010;24(16):1680-1694.
55. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. *Blood*. 2006;107(11):4223-4233.

56. Boudsocq F, Benaim P, Canitrot Y, et al. Modulation of cellular response to cisplatin by a novel inhibitor of DNA polymerase beta. *Mol Pharmacol*. 2005;67(5):1485-1492.
57. Niedernhofer LJ, Lalai AS, Hoeijmakers JH. Fanconi anemia (cross)linked to DNA repair. *Cell*. 2005;123(7):1191-1198.
58. Lord CJ, Garrett MD, Ashworth A. Targeting the double-strand DNA break repair pathway as a therapeutic strategy. *Clin Cancer Res*. 2006;12(15):4463-4468.
59. McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res*. 2006;66(16):8109-8115.
60. Tutt AN, Lord CJ, McCabe N, et al. Exploiting the DNA repair defect in BRCA mutant cells in the design of new therapeutic strategies for cancer. *Cold Spring Harb Symp Quant Biol*. 2005;70:139-148.
61. Sanchez-Perez I. DNA repair inhibitors in cancer treatment. *Clin Transl Oncol*. 2006;8(9):642-646.
62. Hickson I, Zhao Y, Richardson CJ, et al. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res*. 2004;64(24):9152-9159.
63. Syljuasen RG, Sorensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing radiation. *Cancer Res*. 2004;64(24):9035-9040.
64. Ma CX, Cai S, Li S, et al. Targeting Chk1 in p53-deficient triple-negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. *J Clin Invest*. 2012;122(4):1541-1552.
65. Chirnomas D, Taniguchi T, de la Vega M, et al. Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. *Mol Cancer Ther*. 2006;5(4):952-961.
66. Jenkins C, Kan J, Hoatlin ME. Targeting the Fanconi anemia pathway to identify tailored anticancer therapeutics. *Anemia*. 2012;2012:481-483.
67. Szabo CI, King MC. Population genetics of BRCA1 and BRCA2. *Am J Hum Genet*. 1997;60(5):1013-1020.
68. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*. 1994;266(5182):66-71.
69. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*. 2006;25(43):5864-5874.
70. Scully R, Chen J, Ochs RL, et al. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell*. 1997;90(3):425-435.
71. Scully R, Chen J, Plug A, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell*. 1997;88(2):265-275.
72. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A*. 1999;96(20):11364-11369.
73. Yu X, Fu S, Lai M, Baer R, Chen J. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev*. 2006;20(13):1721-1726.
74. Burr KL, Velasco-Miguel S, Duvvuri VS, McDaniel LD, Friedberg EC, Dubrova YE. Elevated mutation rates in the germline of Polkappa mutant male mice. *DNA Repair (Amst)*. 2006;5(7):860-862.
75. Sweasy JB, Lang T, DiMaio D. Is base excision repair a tumor suppressor mechanism? *Cell Cycle*. 2006;5(3):250-259.
76. Sweasy JB, Lauper JM, Eckert KA. DNA polymerases and human diseases. *Radiat Res*. 2006;166(5):693-714.
77. Bagby GC, Olson SB. Cisplatin and the sensitive cell. *Nat Med*. 2003;9(5):513-514.
78. Kennedy RD, Chen CC, Stuckert P, et al. Fanconi anemia pathway-deficient tumor cells are hypersensitive to inhibition of ataxia telangiectasia mutated. *J Clin Invest*. 2007;117(5):1440-1449.

Stephen B. Baylin

5 Epigenetics and Cancer

It has been known for decades that genetic alterations are a fundamental driving force in the initiation and progression of human cancers. It is also now apparent, through a more recent and growing body of work, that epigenetic changes may be equally important in tumor development. *Epigenetics* refers to heritable changes in gene expression in somatic cells that are determined by other than alterations in the primary base sequence of DNA.[1] In essence, the primary sequence of DNA provides the "hard drive," storing all information to determine cell phenotypes. However, to selectively function, DNA requires the "software packaging" of epigenetics to guide patterns of heritable gene expression. In this way, different cell phenotypes emerge against a common DNA sequence background to facilitate processes such as embryonic development and cell differentiation. Just as DNA mutations can mediate individual stages of tumor development by fostering over-, or under-, function of key genes, epigenetic abnormalities can heritably allow similar gene expression aberrations. In this chapter, features are outlined of this latter form of altered gene function and how it is coming to affect the understanding and management of human cancer.

The Molecular Basis for Epigenetic Control of Gene Expression

To specify regions of DNA that contribute to gene expression patterns in a cell, the exposure of sequences to, and the function of, the transcriptional machinery is controlled by packaging of the DNA in the nucleus.[1] This is accomplished, as described later, through a complex and dynamic interaction of DNA with proteins to constitute cellular chromatin, which undergoes posttranslational modifications to establish the transcriptional and regulatory activity of DNA regions and individual genes.[1] This DNA regulation is also modulated by patterns of the only postreplicative modification made directly to DNA, methylation at cytosines located ahead of guanosines (the CpG dinucleotide) in the genome.[1] Alterations to all of these processes are being increasingly identified as important to the evolution of cancer.

The basic molecular unit of DNA packaging, the nucleosome is a structure characterized by the wrapping of approximately 146 bp of DNA around what is termed the *histone octamer* consisting of a tetramer of two histone H3, H4 dimers and a dimer of histone H2.[2] Groups of nucleosomes may in turn be organized into higher order structures through the actions of chromatin remodeling complexes.[3-5] Tightly compacted nucleosome aggregates, or "closed chromatin," are characteristic of DNA regions that are transcriptionally silent, versus more irregularly and linearly spaced nucleosomes, or "open" chromatin, characteristic of DNA regions where transcription is active.[6,7]

One of the most exciting and dynamic areas of chromatin biology concerns another key facet of nucleosome function, posttranslational modification of key amino acid residues of the histones, that helps determine the transcriptional status of genomic regions (Figure 5-1). These modifications constitute what has been termed the *histone code* for gene expression regulation.[8-10] Thus, acetylation of key residues, such as lysine 9 of histone H3 (H3K9acetyl), by enzymes known as histone acetylases (HATs) usually specifies for transcriptionally active regions while deacetylation at these sites, mediated by histone deacetylases (HDACs), is usually associated with transcriptional repression. Methylation of key amino acids also occurs and may be an activating or inactivating mark, depending on the site. For example, the mark of H3K4 methylation is enriched at active areas, whereas H3K9 methylation or H3K27 methylation is characteristic of transcriptionally repressed regions.[8-10] These methylation marks are controlled by a dynamic process involving individual histone methyltransferases, which place the marks, and demethylases, which can remove them.[11-15]

Interacting with all of the dynamics for nucleosome assembly, placement, and histone modifications, to mediate nuclear packaging of DNA, is the modification of DNA methylation (see Figures 5-1 and 5-2). This process, which as noted earlier occurs predominantly in all but embryonic stem cells (ESCs) at CpG dinucleotides, is mediated by three DNA

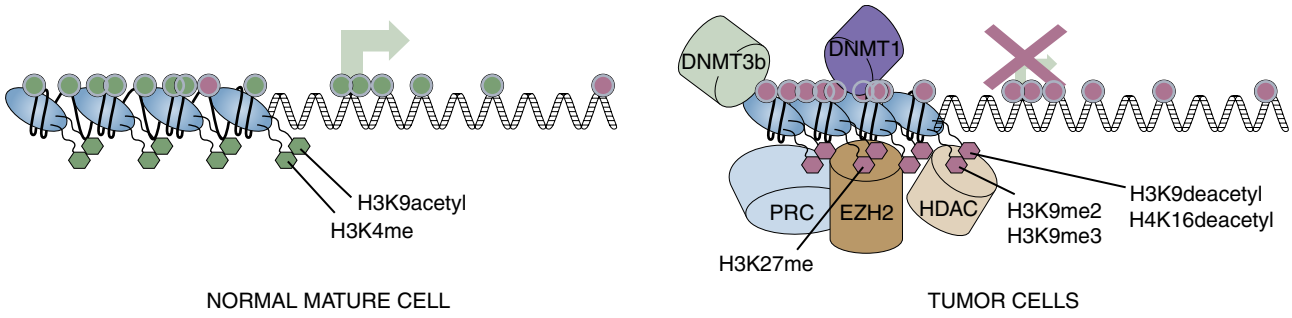


Figure 5-1 Chromatin surrounding an actively expressed gene in a normal mature cell versus surrounding that same gene when it is DNA hypermethylated and aberrantly, heritably, silenced in a tumor cell. On the left, the chromatin is composed of histone modifications associated with active transcription (H_3K_4me) and (H_3K_9acetyl), and the DNA is largely unmethylated at CpG sites *(green circles)* with only occasional methylation (*red circles*). The nucleosomes *(large blue ovals)* are linearly arranged as associated with the areas of active transcription defined in Figure 5-2. The gene on the right is fully transcriptionally repressed *(large red X);* the DNA is methylated and DNA methylating enzymes are present (DNMT1 and -3b); HDACs are present to catalyze histone deacetylation; the machinery of transcriptional repression is present, including the PcG proteins (PRC) with EZH_2, which catalyzes the H_3K_{27}me3 mark *(red hexagons);* and the key silencing marks of H_3K_9me2 and me3 are also present *(red hexagons)*. The nucleosomes are more tightly compacted, as is representative of the repressive chromatin shown in Figure 5-2. *(From Ting AH, McGarvey KM, Baylin SB. The cancer epigenome: components and functional correlates.* Genes Dev *2006;20:3215-3231, with permission.)*

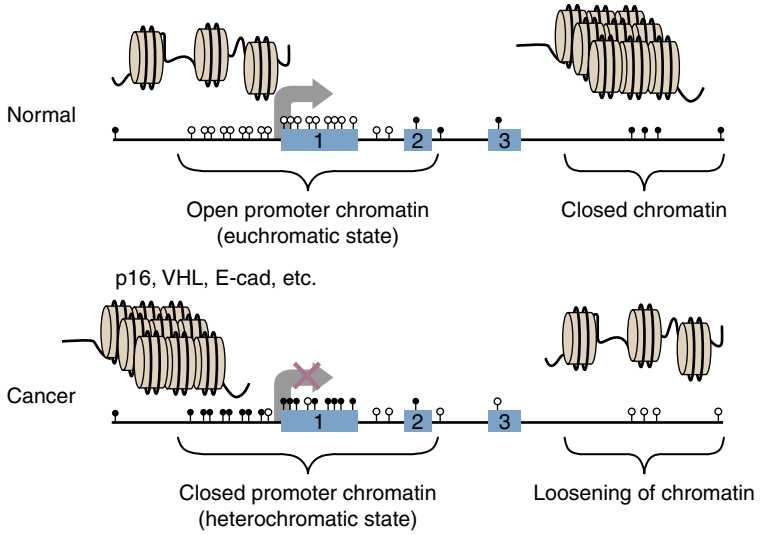


Figure 5-2 The normal versus cancer epigenome *Top:* In normal mammalian cells, CpG islands in proximal gene promoter regions (a three-exon gene is shown, with each exon marked in blue and numbered) are largely protected from DNA methylation (cytosines, *open lollipops*) and reside in restricted regions of open chromatin (*inset*, upstream of transcription start shows three nucleosomes with wide spacing), or euchromatic states, favorable for gene transcription *(large gray arrow)*. In contrast, for most regions of the genome, such as in the bodies of many genes and areas outside genes, particularly including repeat elements and pericentromeric regions, the cytosines in CpG dinucleotides are methylated *(black lollipops)*. This DNA methylation is characteristic of the bulk of the human genome, which is packaged as closed chromatin (the inset above methylated CpGs shows multiple nucleosomes with higher-order, tight compaction) unfavorable for transcription. *Bottom:* In cancer cells, there tends to be a reversal of this pattern. Proximal promoter CpG islands for many abnormally silenced genes (as represented by the same gene as shown in the top panel, which is depicted as representing the tumor suppressor genes listed) become DNA hypermethylated and reside in a closed chromatin, or more heterochromatic-type state, which is not favorable for transcription *(red X)*. In contrast, cytosines in CpG dinucleotides in other regions of the genome display hypomethylation and are associated with states of aberrantly loosened chromatin. The overall result is abnormal chromatin packaging with the potential for underpinning an abnormal cellular memory for gene expression and for conveying abnormal structural function for chromosomes. *(From Ting AH, McGarvey KM, Baylin SB. The cancer epigenome: components and functional correlates.* Genes Dev *2006;20:3215-3231, with permission.)*

methyltransferase (DNMT) enzymes that utilize *S*-adenosylmethionine as a methyl donor group to transfer this moiety for covalent linkage to the cytosines.[6,7] DNA methylation adds a dimension to packaging of DNA and nucleosomes into repressive domains by stabilizing the heritable nature of transcriptional silencing.[6,7] A key aspect of this dimension concerns the distribution of DNA methylation in the genome (see Figure 5-2). In most genomic regions, the CpG dinucleotide is underrepresented because, over evolution, these cytosines have been depleted through deamination of methylcytosines to form thymines.[6,7] However, as many as 80% of these remaining CpG sites are DNA methylated in the human genome, and this has an important functional correlate. This methylation corresponds to the fact that most of our genome, in adult cells, is packaged away into nucleosome-compacted DNA characteristic of regions of transcriptional repression (see Figure 5-2). This may constitute one of the most important functions of genomic DNA methylation, which is to ensure tight

heritability of overall genomic transcriptional repression to prevent unwanted expression of elements such as viral insertions, repeat elements, and other potentially deleterious sequences.[16]

In contradistinction to the depletion of CpGs throughout most of the genome, approximately half of the genes in the genome have regions in their promoters, termed *CpG islands* where the expected frequency of the nucleotide has been preserved (see Figure 5-2). For most such genes, these islands are protected from DNA methylation, and this methylation-free state is associated with active transcription of these genes, or preservation of their being in a transcription-ready state.[6,7,17] These CpG islands are the target of key epigenetic abnormalities in cancer cells, as discussed in detail in subsequent sections of this chapter.

In addition to the previously described role of DNA methylation in global DNA packaging, it is also linked to regulation of expression for specific genes in normal cells. In this regard, when localized to gene promoter regions, it may act to provide a tightening of heritable states for gene silencing. Examples include the imposition of DNA methylation in the promoters of genes shortly after other processes initiate their silencing in regions on the inactive X-chromosome of females.[18] A similar role is apparent in genes that are imprinted in mammals wherein DNA methylation of promoter regions is seen on the silenced allele of such genes.[19,20] DNA methylation also may participate in regulating expression of certain genes in normal cells that are expressed in a tissue-specific manner, such as the silencing of globin genes in all but cells actively engaged in erythropoiesis.[21,22]

In the gene-silencing roles, there is a tight interplay between the modification of key histone amino acid residues and DNA methylation. Thus, at least in lower organisms such as *Neurospora* and *Arabidopsis*, methylation of lysine 9 of histone H3 (H3K9me) may help determine positions where cytosine methylation is placed in the genome.[23,24] Increased levels of the active histone modification H3K4 methylation can be inhibitory to the recruitment of the DNA methylating enzymes.[25] In turn, DNA methylation recruits a series of proteins, methylcytosine binding proteins (MBPs), which are complexed, in turn, with HDACs, which help maintain the deacetylation of H3K9 and other key histone lysines in regions of silenced genes.[6,7]

Abnormalities of DNA Methylation and Chromatin Organization in Cancer: The Cancer "Epigenome"

Overall Characterization

The organization of the genome, as mediated by chromatin and DNA methylation, appears to be quite abnormal in cancer cells of all types when compared with the corresponding cells in normal renewing adult tissues.[26-28] In many cancers, total levels of DNA methylation are decreased with losses apparent within repeat sequences, the bodies and promoters of selected genes, and in the pericentromeric regions of chromosomes.[26-28] The full ramifications of these losses are still being explored, but the changes have the potential for associating with unwanted gene expression and especially, in terms of the pericentromeric abnormalities, with chromosomal instability.[26-30]

Table 5-1 Examples of Pathways Affected by Aberrant Gene Silencing in Cancer

Pathway	Genes
Cell cycle control	*p16, p15*
Apoptosis	*DAP-kinase, ASC/TMS1, HIC1*
Increased stem/developmental pathway activity (Wnt, etc.)	*SFRPs*
DNA damage repair	*MLH_1, O^6-MGM, GST Pi*
Cell adhesion	*E-cadherin*
Cell migration	*TIMPs*
Differentiation	*GATA-4, GATA-5, TGF-β receptor*
Chromosomal stability	*CHFR*

Recently, extensive analyses of DNA methylation patterns in cancer have revealed that, in addition to the regions mentioned previously, there are large megabase regions for loss of DNA methylation scattered over many chromosomes.[31] Within these regions, more localized gains of DNA methylation simultaneously reside in the normally unmethylated CpG islands of promoters of many genes. These methylation gains are, to date, the most studied of the epigenetic abnormalities in cancer[31-33] and are associated with repressive chromatin changes and potentials for aberrant loss of gene expression and function.[26-28,32-34] In fact, it is increasingly apparent that potential disruption of gene function as a consequence of promoter DNA hypermethylation is as frequent, or more frequent, in cancers than mutations as a potential mechanism for loss of tumor suppressor gene function.[26] Individual tumors may actually contain hundreds of such affected genes, which include many of the best characterized tumor suppressor genes[26-28,35] and genes involved with virtually every cellular pathway for which alterations are thought to drive the initiation and progression of cancer[36] (Table 5-1),[26-28,35,37] including those for cell cycle events, apoptosis, developmental biology signal transduction for stem cell function, differentiation, cell-cell adhesion, cell-cell recognition, cell migration and invasion, and others.[33-35,37] The list of involved genes, as identified by study of candidate genes and techniques for randomly screening the cancer epigenome,[33-35,38,39] is steadily growing for virtually all major cancer types.

Chromatin Abnormalities in Cancer and Interplay with DNA Methylation Changes

In addition to abnormalities in DNA methylation in cancer, chromatin alterations are also frequent, and there can be interplay between the two. Indeed, one of the most active areas of cancer epigenetics research at present, and one of utmost importance to the translational impact for cancer prevention, diagnosis, and treatment, concerns delineation of the molecular underpinnings of how the cancer epigenome evolves.[32] This investigation has benefited from, and contributed to, the explosion of knowledge over the past 5 to 10 years in understanding how chromatin functions for packaging of the genome and for modulation of gene expression.[40] Although many remain to be elucidated, important findings are emerging that provide clues to the origins of epigenetic abnormalities in cancer.

The initiation of DNA methylation, its maintenance, and its role in transcriptional repression are all dependent on its interaction with chromatin organization (see Figure 5-1). As previously alluded to, the sites of DNA methylation themselves may be dependent, initially, on histone modifications. Thus, H3K9 methylation, and the histone methyltransferases that catalyze this mark, appears required for DNA methylation in lower organisms such as *Arabidopsis* and *Neurospora*.[23,24] In addition, the polycomb group of proteins,[41-43] discussed in more detail later, which target another key gene repression mark to nucleosomes, H3K27me, have been implicated in the targeting and maintenance of DNA methylation. Also, a series of proteins called methylcytosine binding proteins (MBPs), and the protein complexes in which they reside, can bind to methylated CpG sites to help relay, and/or maintain, a silencing signal.[6,7] These complexes contain the previously mentioned enzymes, histone deacetylases (HDACs), which catalyze the deacetylation of key amino acid residues, such as H3K9, that are highly characteristic of transcriptionally silent regions of DNA.[6,7,44] The DNMTs themselves also interact with HDACs to help target these enzymes to sites of DNA methylation.[45-47]

The alterations in the levels or ratios of factors that mediate epigenetic abnormalities in cancer cells are first manifest by certain global abnormalities. Thus, increases in the levels and activities of the DNA methylation catalyzing enzymes[48]; of the proteins in complexes that modulate the enzymes that catalyze transcriptional repression histone modifications[49-51]; and altered levels of the repressive histone marks themselves, including loss of acetylation at H4K16 and increased levels of H4K20 acetylation,[52] are all reported as common hallmarks of human cancer. Locally, at gene promoters affected by promoter DNA methylation and aberrant gene silencing (see Figure 5-1), there are decreases in histone modifications associated with active gene transcription, such as acetylation of H3K9 and H4K16; increases in modifications associated with transcriptionally repressive chromatin, including H3K9me2 and me3 and H3K27me3; and increases in the enzymes that catalyze these latter repressive marks.[48,53]

The precise manner in which all of these chromatin components interact to initiate and/or maintain abnormal gene promoter DNA methylation and the attendant silencing of involved genes is not yet known. As noted earlier, in cancer, these gains of DNA methylation can occur as focal changes within large regions of loss of normal DNA methylation.[31] These data suggest that molecular maintenance of chromatin and DNA methylation boundaries "break down" during tumor progression. Factors such as "insulator" proteins, which maintain separation between transcriptionally repressive and active chromatin states, are altered and/or chromatin states that associate with such transcription states and are also shifted.[32]

How might such chromatin alterations come about? One potential mechanism concerns recent exciting findings stemming from deep sequencing analyses of most solid and liquid tumor types that are revealing many frequent mutations in genes encoding for proteins that normally ensure formation of chromatin in normal cell epigenomes[32,54] (see Figure 5-2 and Table 5-1). The high frequency of these changes suggests that they are fundamentally important to the initiation and progression of cancer[32,54] and may justify considering their roles as "driver" mutations. As such, they would contribute important steps in the initiation and/or progression of cancer. A major challenge, however, is to understand the exact consequences of these mutations for key steps in tumorigenesis and for their precise contribution to cancer-specific alterations of chromatin and DNA methylation. One mutation particularly, in the IDH1 and 2 genes in glioblastomas and leukemias,[55-57] appears to cause a metabolic balance that inhibits enzymes that remove key gene expression silencing histone marks and/or that can remove DNA methylation.[58,59] These mutations, when studied experimentally, appear to disrupt normal stem/progenitor cell function and commitment of cells to proper lineages. Moreover, the tumors that harbor them have an increased frequency of abnormal promoter region CpG island DNA methylation.[60,61]

The studies just referred to concerning regional changes in epigenetic patterns in cancer suggest that many genes with CpG island–containing promoters are particularly vulnerable to adopting abnormal DNA methylation during the abnormal cellular expansion that underlies the earliest phases of tumor progression[48,62-64] (Figure 5-3). A growing number of studies reveal that these genes are enriched for those that are important for developmental functions.[65] Importantly, in normal embryonic and adult stem cells, these genes are

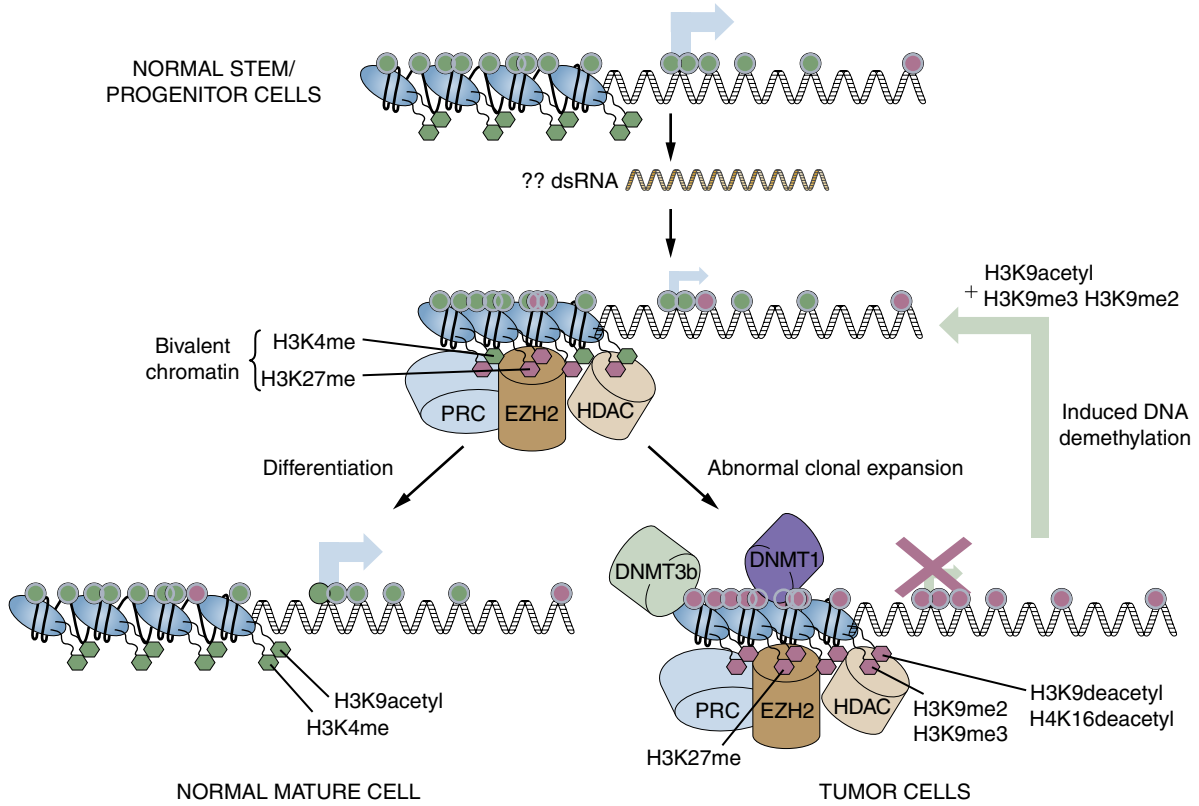


Figure 5-3 A model for the potential contribution of stem cell chromatin to the initiation and maintenance of aberrant epigenetic gene silencing in cancers During normal ES cell formation, a bivalent chromatin is recruited to the promoters of a subset of genes that need to be held in a low-expression state to prevent lineage commitment. The involvement of small interfering RNA (siRNA) species could be a trigger to this process, and the chromatin is composed of histone modifications associated with active transcription (H3K4me) and inactive transcription (H3K27me). The PcG proteins (PRC) are responsible for the H3K27me3 mark through the HMT, EZH2, and deacetylation of key histone lysine residues is catalyzed by HDACs that are recruited by multiple transcriptional repressive complexes. At such genes, DNA is largely unmethylated *(green circles),* and histones may be maintained in a mixture of acetylated *(green hexagons)* and deacetylated *(red hexagons)* states. *Bottom left:* With normal cell differentiation and lineage commitment, the genes become transcriptionally active, and the silencing marks are reduced while active histone marks are retained. DNA remains unmethylated. However, as shown at *bottom right,* during cancer-predisposing events, abnormal pressure for stem/progenitor cell proliferation with retained bivalent chromatin may allow polycomb proteins and/or marks to recruit other silencing marks such as H3K9me2 and H3K9me3 and DNA methyltransferases. The promoter evolves abnormal DNA methylation *(red circles)* and a tight heritable gene silencing *(large red X),* which results in loss of function for genes. Tumors may arise in such clones with subsequent progression steps. Experimentally, the potential underlying bivalent chromatin for such tumor genes, plus retained H3K9me3, can be revealed by induced DNA demethylation *(large green arrow)* and resultant gene re-expression. *(From Ting AH, McGarvey KM, Baylin SB. The cancer epigenome: components and functional correlates.* Genes Dev *2006;20:3215-3231, with permission.)*

maintained in a low, poised transcription state in which their promoter regions are marked, not by DNA methylation, but by a "bivalent" chromatin pattern characterized by a broad distribution of the PcG-mediated H3K27me3, repressive histone modification accompanied by a more narrow zone of the active mark, H3K4 methylation surrounding the transcription start site.[48,62-64] This bivalency may allow regulatory flexibility by keeping these genes in a low, poised quiescent transcription state to maintain stem cell pluripotency and/or prevent their undergoing premature lineage or differentiation commitment.[65] Interestingly, these genes in ESC typically reside in the large regions mentioned earlier that broadly lose DNA methylation in cancer but gain CpG island methylation focally around promoter regions.[31] Thus, it appears that for these vulnerable genes, during tumorigenesis, there is imposition of abnormal, promoter CpG island DNA methylation as opposed to the control of expression of these genes in normal cell renewal by a balance involving polycomb proteins and active gene histone marks. The more stabilizing silencing process in association with DNA methylation may, then, provide a mechanism by which inability to induce at least some of the foregoing genes could help foster maintenance of stemlike cells and/or a block in their ability to differentiate.[65,66]

Relationships of Epigenetic Changes in General, and Aberrant Gene Silencing in Particular, to the Progression of Cancer

Although losses and gains of DNA methylation in cancer may arise at any point during tumor progression, it has become apparent that many of the changes arise early, before

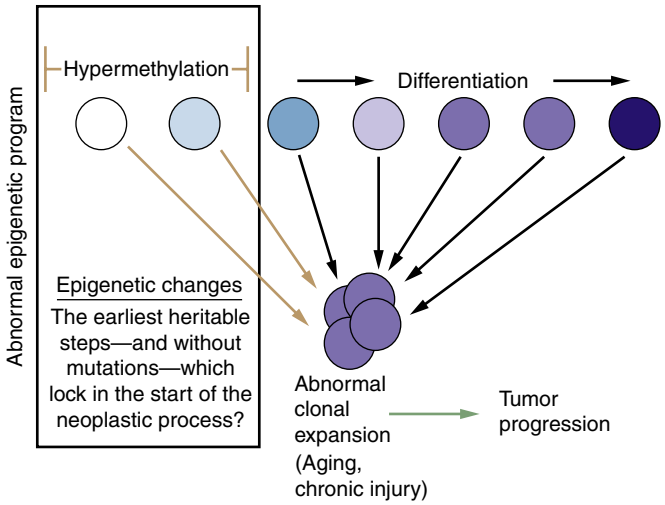


Figure 5-4 The potential that epigenetic gene-silencing events have for participation in the earliest stages of tumor progression As discussed in the text and Figure 5-3, suppression of gene transcription can be a normal event for a group of key genes in stem cells and progenitor cells as adult epithelial-cell renewal takes place *(left large box)*. This low-level gene expression is accomplished by a balance of chromatin modifications that associate with active and repressed transcription (bivalent chromatin; see Figure 5-3), but transcription can increase in maturing cells during normal cell renewal. This balance of control for gene expression allows stem and progenitor cells to progress along a normal differentiation pathway (moving with *arrow* from left to right across the top of the figure). During chronic and abnormal pressures on stem-cell and progenitor-cell pools for tissue repair, there is a tendency for the gene chromatin constituents in these cells (see Figure 5-3) to recruit promoter DNA hypermethylation *(top of large box)*, and this becomes associated with heritable silencing of the genes (abnormal epigenetic program, *large box*). This inability of the genes to increase with maturation cues facilitates abnormal clonal expansion of stem/progenitor cells *(heavier arrows)*, at the expense of differentiation. Such expansion may occur in stroma, leading to an abnormal environment that helps support epithelial tumor growth. This process renders the abnormal clones at risk for further tumor progression *(bottom arrow)* driven by subsequent genetic or epigenetic events.

frank carcinomas.[34,37,48,67] In fact, it is possible that some of the events, such as silencing of key genes, could even initiate the abnormal clonal expansion that creates early preinvasive lesions, which are then at risk for subsequent genetic and epigenetic events that further tumor progression and lead to invasive and metastatic cancer (Figure 5-4).[34,37,48,67] The genes silenced, or groups of such genes, may provide loss of tumor suppressor function that allows cells to abnormally survive the hostile environments that are risk factors for cancer development, such as chronic inflammation, and expose cells to DNA-damaging agents such as reactive oxygen species (ROS). In fact, experimental data now indicate that increases in ROS can rapidly trigger localization of protein complexes, including DNA methyltransferases, to CpG islands in gene promoters.[68] During this period, for the types of low-transcription genes that, as discussed in a previous section, seem vulnerable to adopting abnormal promoter DNA methylation, abnormal DNA methylation begins to appear.[68] This change in key groups of genes during injury repair may enable cells that would normally undergo apoptosis from DNA damage to survive and expand. They may, then, more easily select for mutations and/or chromatin damage that may favor subsequent tumor progression (see Figure 5-4).

There are now several key examples of this proposed early role for DNA hypermethylation and gene silencing in tumor progression. One of the major tumor suppressor genes in cancer, where loss of function leads to cell cycle abnormalities and uncontrolled growth, is *p16*.[69] A role for this loss of function in early tumorigenesis, via early expansion of stem cells, would be predicted from data in *p16* knockout mice revealing that germline loss of this gene can increase stem-cell lifespan.[70-72] The rate of point mutations in *p16* in most cancer types is low, but the gene is a frequent target for early methylation in these same tumors, such as breast cancer and non–small-cell lung cancer (NSCLC).[73,74] This methylation occurs early in tumor progression, before invasive cancer.[73,75] In fact, histologically normal mammary epithelium from some healthy women without malignancy can harbor focal *p16* promoter hypermethylation.[76] Experimentally, early loss of *p16* in mammary epithelial cells precedes genomic and epigenetic instability.[77-79] A recent study in the Cancer Genome Atlas project (TCGA) further emphasizes how, in the squamous form of NSCLC, abnormal methylation of p16 is mutually exclusive to mutations of the gene.[80]

Another excellent example of the potential role for early epigenetic abnormalities and stem/precursor cell expansion to contribute to early steps in tumor progression involves colon cancer. In this disease, cancer risk can begin with the appearance of aberrant crypt foci in the colonic epithelium, and these harbor premalignant, hyperplastic, preadenomatous cells.[81,82] The evolution of colon cancer is highly dependent on abnormal activation of the stem/precursor cell Wnt pathway, which by the time frank polyps and/or invasive lesions appear, is driven by classic inactivating mutations in the *APC* gene or activating mutations of β-catenin, key downstream players in the pathway.[83,84] In aberrant crypt foci, however, such mutations may not be present, yet there is DNA hypermethylation[37,85] of a family of genes, the SFRPs, which encode for membrane region proteins that antagonize Wnt interaction with its receptors.[85,86] This hypermethylation persists throughout colon tumor progression and can later collaborate with the downstream mutations in driving the Wnt pathway.[37,85]

Translational Implications of Epigenetic Changes in Cancer

The delineation of epigenetic abnormalities in tumorigenesis is now actively contributing not only to our understanding

of the biology of cancer but also to potentially new ways for managing these diseases. First, the overall abnormalities in chromatin organization and DNA methylation provide potential biomarkers for use in cancer risk assessment, early diagnosis, and prognosis assessment. Second, the molecular features that contribute to the epigenetic abnormalities in cancer are increasingly offering new targets for devising novel therapy strategies for all types of cancers. Some of the progress in these arenas is reviewed in this section.

Epigenetic Changes and Development of Biomarker Strategies

Overall and local chromatin changes in cancer provide potential markers for cancer management. For example, during the early stages of tumor progression, some of the histone modifications altered in cancer cells (see Figures 5-1 and 5-3) are manifest, as previously discussed. These can be global in tumor cells such that levels of these parameters, including losses of monoacetylated and trimethylated forms of histone H4 and losses of acetylated Lys16 and trimethylated Lys20 residues of histone H4, reflect either the presence of cancer or its stages.[52,87] Changes in modification marks on histones H3 and H4 have been correlated with aggressiveness of prostate cancer.[87] These global changes are hypothesized to be common hallmarks of human tumor cells and hold promise for the development of important biomarkers. Similarly, increases in levels of enzymes that catalyze key facets of cancer epigenetic abnormalities, such as the DNA methyltransferases for DNA methylation[48] and, more recently, histone methyltransferases such as EZH2[50,51] or H3K27 methylation, and other PcG gene silencing constituents,[88] have been correlated with several cancer types and correlated with aggressive behavior.

The most developed biomarker strategies have been centered on the gene promoter DNA hypermethylation and gene silencing. The use of promoter-hypermethylation sequences as a molecular signature is providing one of the most promising biomarker strategies for cancer.[89-91] One advantage of the approaches being adopted relies on the relative stability of DNA as compared with many proteins and RNA, which allows for use of paraffin-embedded clinical samples for detection strategies. Given the fact that, as discussed, the numbers of genes DNA hypermethylated is so high in individual tumors, and that this phenomenon is common in all cancer types, it is not difficult to build profiles of relatively small hypermethylated gene panels in which one or more markers are positive in virtually any cancer.[92] Combined with a repertoire of sensitive polymerase chain reaction (PCR)-based assay procedures to specifically detect the hypermethylated sequences,[90,91,93] and the fact that these assays can be targeted to constant positions of the abnormal CpG methylation in gene promoter regions, relatively simple detection strategies are being constructed and now include adaptation of the assays to nanoparticle-based platforms.[94,95] With such assays, abnormally methylated gene sequences have been detected in sources as diverse as DNA extracted from tumor, lymph nodes, serum, sputum, bronchial-lavage fluid, and urine for patients with all varieties of cancer types.[89-91] The strategies range from determining whether the methylation patterns in tumors reflect prognosis for behavior, to use of marker detection in distal sites for purposes of cancer risk assessment, early diagnosis, and staging. For example, studies of sputum DNA from patients at high risk for lung cancer have found that invasive tumors may be predicted, with odds ratios of 6 or more, more than a year before clinical detection of cancer,[96] and findings of abnormal methylation markers in sputum may be useful for predicting which patients with surgically resected early-stage lung cancers may experience a recurrence.[97] The occurrence of hypermethylation of specific genes in tumor DNA may predict future behavior of a cancer; reportedly, this change for the *p16* gene in DNA from lung cancers predicts high likelihood of poor outcome.[98] Assays to detect DNA hypermethylation of the GST-pi gene in needle biopsies of the prostate are reaching clinical use as an aid to refine the diagnosis of prostate cancer. Recently, simultaneous detection of abnormal promoter DNA methylation of a panel of four genes, in DNA from tumor and chest lymph nodes assessed to be microscopically free of tumor by pathology exam, provides a potential strategy for predicting recurrence in early-stage non–small-cell lung cancer (NSCLC).[99] Similarly, sensitive detection of these types of gene promoter DNA methylation abnormalities in stool and/or serum DNA appears promising for the early detection of colon polyps and/or cancer.[100,101]

The promise of these biomarker approaches will be realized only through continued studies of ever-increasing size. The precise assays best suited for routine clinical use must be determined, and approaches that build the most quantitative determinations into these assays, increasingly being applied,[102] must be evaluated. Confounding issues must be continuously considered. A most critical one is to always consider whether the presence of hypermethylated gene markers in normal-appearing tissue settings means cancer risk as opposed to actual cancer presence. This accentuates the importance of the information discussed earlier in this chapter concerning the biology of cancer as it involves epigenetic abnormalities. The position for appearance of individual gene markers in tumor progression is critical and must be paired with consideration of risk factors. For example, gene promoter methylation in normal tissues can increase with age, as best studied in the colon,[103] and

parallels the risk of cancer at a given site. All of this information defines the potential power of marker strategies using gene promoter hypermethylated sequences and the caveats that must be considered in using these strategies.

Perhaps one of the most promising uses for gene hypermethylation markers, and another that is now close to general clinical realization, concerns their use for prediction of drug sensitivity. This strategy exploits the fact that aberrantly silenced genes involved with this epigenetic abnormality can belong to pathways that dictate cellular pathways integral to drug responsiveness. The most developed example of this is the silencing of the DNA repair gene *O⁶-MGMT*, which encodes for a protein that mediates removal of bulky alkylation adducts from guanosines.[89] Several tumor types lose the function of *O⁶-MGMT* via aberrant silencing of the gene, and constituent cells have a diminished capacity to repair alkylation damage, rendering them sensitive to alkylating agents such as temozolomide.[104,105] Thus, multiple studies reveal that patients with brain tumors harboring *O⁶-MGMT* respond remarkably better to this agent than those whose tumors lack this change, providing an exceptionally promising marker to stratify patients with this lethal tumor type for best therapy approaches.[105,106] If ongoing trials continue to validate this, a relatively easy and rapid marker will be available for routine clinical use.

Targeting Epigenetic Abnormalities for Cancer Prevention and Therapy

There is now growing appreciation that our expanding knowledge of epigenetic abnormalities in cancer, in general, and most especially, at present, the definition of aberrant gene silencing as an alternative mechanism to mutations for loss of tumor suppressor gene function, offers extraordinary potential for exploitation in managing cancer.[89,107] First, there is the critical difference that, as compared with mutations, epigenetic gene silencing, as we have discussed in this chapter, is potentially reversible. Second, the growing list of molecular steps being defined as components of the silencing offers more individual and combinatorial targets for considering interventions. Third, the early position of aberrant genes in tumor progression makes reversal of the silencing an attractive target for prevention approaches. Also, the potential of the silenced genes to participate in tumor recurrence suggests that the adjuvant treatment arena may be an attractive area for epigenetic therapy. Fourth, and perhaps most important, the biology discussed in this chapter, including the high frequency of the gene silencing abnormality in all cancer types, the numbers of genes involved in individual tumors, and the critical pathways for cancer development in which the involved genes participate, makes reversal of gene silencing not only a rational target for therapy, but an essential one to consider (Figure 5-5). If successful, reversal of the entire gene silencing in a given patient's tumor could, with one targeted therapy approach, reverse virtually every key signal pathway involved in the initiation, progression, and maintenance of the cancer (see Table 5-1 and Figure 5-5).

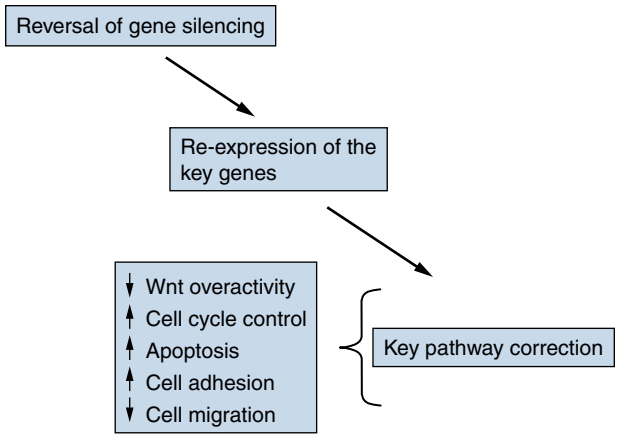


Figure 5-5 The theory behind emphasizing targeting reversal of aberrant gene silencing as a strategy for cancer prevention and therapy The concept is depicted that, based on the numbers of epigenetically silenced genes in a given tumor, the numbers of pathways affected by the epigenetically mediated loss of gene function, and the network effects of the silencing within and between the pathways, the strategy of reactivating silenced genes presents a unique opportunity to counter, via a single therapy, virtually all the steps that drive tumorigenesis.

Where do we stand in this important cancer prevention/therapy endeavor? Indeed, drugs that reverse DNA demethylation, such as 5-azacytidine and 5-aza-2′-deoxycytidine, and histone deacetylase inhibitors that target the histone deacetylation component of gene silencing are already in the clinic[89,106-108] and approved by the U.S. Food and Drug Administration (FDA) for the preleukemic disorder myelodysplasia (MDS) and cutaneous T-cell lymphoma and peripheral T-cell lymphomas,[109,110] respectively. The concept that initial use of azacytidines followed by administration of histone deacetylase inhibitors (HDACis) may be synergistic for inducing reexpression of aberrantly silenced cancer genes is receiving attention and encouraging early clinical results. It must be stressed, however, that it remains to be established to what degree the individual or combined effects of these drugs on their targets, DNMTs and HDACis, plays a role in their therapeutic efficacy in patients with MDS, related leukemias, and cutaneous lymphomas for the HDACis.[89,107,111-115] Encouraging results indicate that at least some of the clinical effects are due to true reversal of epigenetic targets. First, clinical efficacy is being accomplished, especially for the azacytidines, at far lower doses than the ones initially used. This greatly reduces the toxic effects that may be due to nonepigenetic effects of the drugs, such as DNA damage.[116,117] Indeed, recent preclinical studies indicate

that transient exposure of leukemia and solid tumor cells to low-nanomolar doses of the foregoing DNA-demethylating drugs, without initial cell killing, provide a cell-reprogramming–like effect that can give long-term blunting of cell tumorigenicity and self-renewal capacities.[118]

Second, emerging data suggest that the efficacy of the azacytidines correlates with the acute reversal of gene silencing. In the preclinical studies just mentioned, the low doses of the DNA-demethylating agents can reduce overall, and abnormal gene promoter, DNA methylation with simultaneous reexpression of tumor suppressor genes such as the cyclin-dependent kinase inhibitor encoding gene, *p15*, in leukemia cells.[118] Early reactivation in MDS and leukemias appears to correlate with subsequent patient responses in one study,[111] although others have not found such correlation even though the gene is clearly reexpressed in patients' tumor cells during acute drug treatment.[119] In another study, combination decitabine and trichostatin A, an HDACi, resulted in decreased expression of the multidrug resistance transporter ABCG2 as well as markers of enhanced self-renewal populations in ovarian cancer cells, and increased sensitivity to cisplatin in vivo.[120]

Despite these encouraging developments, much remains to be done if epigenetic therapies are to make a powerful impact on the prevention and treatment of cancer. First, little efficacy for the common solid tumors has been shown. However, most attempts to treat these tumors occurred before it was appreciated that lower, and less toxic, doses of drugs such as the azacytidines and HDACis can be used. The time is ripe for the regimens showing such promise in the liquid tumors to be applied to the treatment of solid tumors. In this regard, a low-dose regimen of 5-azacytidine (Vidaza) plus a histone deacetylase inhibitor, entinostat, has achieved very promising results in a clinical trial for the world's most deadly malignancy, multiply treated, advanced NSCLC.[121] Some 3% of the patients achieved high-grade tumor regression that was durable from nearly 3 to 4 years. Moreover, some 20% of the patients exhibited unusually good, durable tumor responses to subsequent therapies even after short courses of the epigenetic therapy.[121] These are all unusually good response trends in advanced NSCLC and, if verified in subsequent clinical trials, would provide an important role for epigenetic therapy in the management of solid tumors. In this regard, several groups have now reported the use of demethylating agents to restore platinum sensitivity in platinum-resistant ovarian cancer. Bast and colleagues reported a phase I/II study of azacitidine and carboplatin showing durable responses and stable disease (median duration of therapy 7.5 months) in 46% of platinum-resistant or refractory ovarian cancer patients.[122] A similar study using a demethylating agent and carboplatin in the same patient population reported a 40% 6-month progression-free survival, with one patient having a complete response.[123] Larger trials testing the use of demethylating agents to overcome platinum resistance are presently ongoing.

Along with the foregoing progress and potential for use of DNA demethylating agents, much work is still needed to optimize the approaches. New classes of inhibitors of the DNMTs may be needed that do not incorporate into DNA and, for ease of patient use, can be administered orally. Also, as discussed previously in this chapter, our increasing knowledge of the chromatin components of gene DNA hypermethylation-associated gene silencing must be exploited. As shown in Figure 5-3, the retention of key silencing chromatin marks for reexpressed genes following promoter DNA demethylation predicts that, as experimentally seen,[53,124] once administration of drugs such as the azacytidines is stopped, the silencing will return. Thus, feasibility for prolonged drug regimens may need to be shown and, indeed, such chronic administration appears possible for the azacytidines.[114,115] Finally, the other chromatin components of the aberrant gene silencing represent additional drug targets that may enrich therapy possibilities.

As previously mentioned, HDACis are the other major class of epigenetic modulating agents that have been tested and approved for treatment of malignancy. As single agents, their activity has been limited to the lymphoma disorders for which they are now FDA approved.[125] Nevertheless, an emerging series of preclinical studies and clinical trials suggest that HDACis could be important drugs for improving cancer management. One recent example is the suggestion that HDACis may be able to inhibit subpopulations of cancer cells that drive tumorigenesis and are usually resistant to most therapies. The precise definition of these "stem-cell–like" cells is still controversial, but the evidence is strong that most cancers harbor subpopulations, to a variable percentage, of such cells.[126-128] In this regard, although resistance to chemotherapy and other therapies may often result from new mutations that emerge during the course of treatment, epigenetic control may also come into play. Settleman and colleagues recently reported that resistance to treatment of multiple types of cancer cells, with both targeted therapy drugs and chemotherapy agents, can be involved with drug-tolerant, stemlike cells on an epigenetic basis.[129] Low doses of several HDACis could reverse this drug resistance.[129] These findings suggest that a potential use for HDACis is reversing or delaying drug resistance. Indeed, this potential may be emerging in clinical trials. In a randomized phase II trial, combination therapy with the HDACi entinostat plus the epidermal growth factor inhibitor erlotinib provided survival benefit in a subset of the patients with baseline high tumor levels of E-cadherin. This same drug, in a phase II trial for advanced breast cancer, also increased survival when combined with an aromatase blocker.[130] It is important to note

that the exact mechanisms involved in the potential efficacy of HDACis in these trials remain to be determined, and, of course, the results must be validated in trials to follow.

In addition to the work with epigenetic therapy agents that are already in clinical trials, the future is very bright for the use of new agents that target other molecular steps that control the epigenome. In fact, the emerging clinical potential of existing epigenetic drugs, and the explosion in knowledge about chromatin and DNA methylation biology, has led most large pharmaceutical companies to establish programs in developing compounds that target epigenetic abnormalities in cancers. For example, whereas the HDACis just discussed target one class of these enzymes, another class that includes the deacetylase SIRT1 may lie downstream of the abnormal DNA methylation.[131] Thus, inhibition of the activity of this protein appears to cause reactivation of aberrantly silenced cancer genes without the necessity for removal of the promoter DNA hypermethylation.[131] A very exciting development is targeting of the DOT1L protein, which mediates the effects of MLL translocations in leukemia. A small molecule inhibitor has selective antitumor effects on mixed-lineage leukemia (MLL) cells.[132,133] Clinical trials with this type of drug should be forthcoming soon. Also, inhibitors of BRD4, a protein containing domains that recognize acetylated histone lysines, are generating much excitement.[134-136] This protein appears to be a key regulator for the activation of the pervasive oncogene c-MYC and its targets.[134-136] In preclinical studies, BRD4 inhibitors have selective antitumor effects on MLL-fusion leukemias with blocking of c-MYC overactivity.[137,138] These drugs will shortly be appearing in clinical trials.

It is thus apparent that our knowledge of chromatin biology is already being robustly exploited to develop novel approaches to cancer therapy. The era is an exciting one for realizing the major impact on cancer control from targeting epigenetic abnormalities involved in the initiation and progression of cancer.

Key References

1. Allis C, Jenuwein T, Reinberg D. *Epigenetics*. vol. 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2007.
4. Li H, Ilin S, Wang W, et al. Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. *Nature. Jul 6*. 2006;442:91-95.
5. Kingston R, Tamkun JW. Transcriptional regulation by Trithorax group. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:231-248.
8. Jenuwein T. The epigenetic magic of histone lysine methylation. *FEBS J*. 2006;273:3121-3135.
11. Kubicek S, Schotta G, Lachner M, et al. The role of histone modifications in epigenetic transitions during normal and perturbed development. *Ernst Schering Res Found Workshop*. 2006:1-27.
13. Forneris F, Binda C, Vanoni MA, Mattevi A, Battaglioli E. Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process. *FEBS Lett*. 2005;579:2203-2207.
14. Tsukada Y, Fang J, Erdjument-Bromage H, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature*. 2006;439:811-816.
15. Yamane K, Toumazou C, Tsukada Y, et al. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell*. 2006;125:483-495.
18. Brockdorff N, Turner BM. Dosage compensation in mammals. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:321-340.
19. Bartolomei MS. Epigenetics: role of germ cell imprinting. *Adv Exp Med Biol*. 2003;518:239-245.
23. Tamaru H, Selker EU. Synthesis of signals for de novo DNA methylation in *Neurospora crassa*. *Mol Cell Biol*. 2003;23:2379-2394.
24. Jackson JP, Johnson L, Jasencakova Z, et al. Demethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma*. 2004;112:308-315.
25. Ooi SK, Qiu C, Bernstein E, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature*. 2007;448:714-717.
28. Feinberg AP. The epigenetics of cancer etiology. *Semin Cancer Biol*. 2004;14:427-432.
31. Berman BP, Weisenberger DJ, Aman JF, et al. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet*. 2012;44:40-46.
32. Baylin SB, Jones PA. A decade of exploring the cancer epigenome—biological and translational implications. *Nat Rev Cancer*. 2011;11:726-734.
33. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
34. Baylin SB, Jones PA. Epigenetic determinants of cancer. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:457-476.
35. Esteller M. Epigenetics in cancer. *N Engl J Med*. 2008;358:1148-1159.
37. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer*. 2006;6:107-116.
39. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer*. 2005;5:223-231.
40. Ecker JR, Bickmore WA, Barroso I, Pritchard JK, Gilad Y, Segal E. Genomics: ENCODE explained. *Nature*. 2012;489:52-55.
41. Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu Rev Genet*. 2004;38:413-443.
42. Fujimura Y, Isono K, Vidal M, et al. Distinct roles of Polycomb group gene products in transcriptionally repressed and active domains of Hoxb8. *Development*. 2006;133:2371-2381.
43. Grossniklaus U, Paro R. Transcriptional silencing by polycomb group. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:211-230.
48. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome—components and functional correlates. *Genes Dev*. 2006;20:3215-3231.
49. Kirmizis A, Bartley SM, Farnham PJ. Identification of the polycomb group protein SU(Z)12 as a potential molecular target for human cancer therapy. *Mol Cancer Ther*. 2003;2:113-121.
50. Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A*. 2003;100:11606-11611.
52. Fraga MF, Ballestar E, Villar-Garea A, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet*. 2005;37:391-400.
53. McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB. Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. *Cancer Res*. 2006;66:3541-3549.
54. De Carvalho DD, Sharma S, You JS, et al. DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer Cell*. 2012;21:655-667.

55. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. *J Mol Cell Biol*. 2009;1:82-92.
56. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med*. 2009;360:765-773.
57. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med*. 2009;361:1058-1066.
58. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature*. 2012;483:474-478.
59. Sasaki H, Setoguchi T, Matsunoshita Y, Gao H, Hirotsu M, Komiya S. The knock-down of overexpressed EZH2 and BMI-1 does not prevent osteosarcoma growth. *Oncol Rep*. 2010;23:677-684.
60. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553-567.
61. Noushmehr H, Weisenberger DJ, Diefes K, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*. 2010;17:510-522.
62. Widschwendter M, Fiegl H, Egle D, et al. Epigenetic stem cell signature in cancer. *Nat Genet*. 2007;39:157-158.
63. Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet*. 2006.
64. Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet*. 2007;39:237-242.
65. Easwaran H, Johnstone SE, Van Neste L, et al. A DNA hypermethylation module for the stem/progenitor cell signature of cancer. *Genome Res*. 2012.
66. Baylin SB. Stem cells, cancer, and epigenetics. *Stem Book*. 2009:2-14.
67. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet*. 2006;7:21-33.
68. O'Hagan HM, Wang W, Sen S, et al. Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG islands. *Cancer Cell*. 2011;20:606-619.
70. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16(INK4a). *Nature*. 2006.
71. Krishnamurthy J, Ramsey MR, Ligon KL, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature*. 2006;443:453-457.
72. Molofsky AV, Slutsky SG, Joseph NM, et al. Increasing p16(INK4a) expression decreases forebrain progenitors and neurogenesis during ageing. *Nature*. 2006;443:421-426.
76. Holst CR, Nuovo GJ, Esteller M, et al. Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. *Cancer Res*. 2003;63:1596-1601.
79. Reynolds PA, Sigaroudinia M, Zardo G, et al. Tumor suppressor P16INK4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells. *J Biol Chem*. 2006;281:24790-24802.
80. Hammerman PS, Hayes DN, Wilkerson MD, et al. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012;489:519-525.
85. Suzuki H, Watkins DN, Jair KW, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet*. 2004;36:417-422.
87. Seligson DB, Horvath S, Shi T, et al. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature*. 2005;435:1262-1266.
88. Cao R, Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell*. 2004;15:57-67.
89. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349:2042-2054.
90. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer*. 2004;4:707-717.
91. Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer*. 2003;3:253-266.
94. Bailey VJ, Easwaran H, Zhang Y, et al. MS-qFRET: A quantum dot-based method for analysis of DNA methylation. *Genome Res*. 2009;19:1455-1461.
95. Li M, Chen WD, Papadopoulos N, et al. Sensitive digital quantification of DNA methylation in clinical samples. *Nat Biotechnol*. 2009;27:858-863.
96. Belinsky SA, Liechty KC, Gentry FD, et al. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer Res*. 2006;66:3338-3344.
97. Machida EO, Brock MV, Hooker CM, et al. Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. *Cancer Res*. 2006;66:6210-6218.
98. Gu J, Berman D, Lu C, et al. Aberrant promoter methylation profile and association with survival in patients with non-small cell lung cancer. *Clin Cancer Res*. 2006;12:7329-7338.
99. Brock MV, Hooker CM, Ota-Machida E, et al. DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med*. 2008;358:1118-1128.
100. Glockner SC, Dhir M, Yi JM, et al. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res*. 2009;69:4691-4699.
101. Zou H, Allawi H, Cao X, et al. Quantification of methylated markers with a multiplex methylation-specific technology. *Clin Chem*. 2012;58:375-383.
105. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352:997-1003.
106. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov*. 2006;5:37-50.
107. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004;429:457-463.
108. Marks PA, Richon VM, Miller T, Kelly WK. Histone deacetylase inhibitors. *Adv Cancer Res*. 2004;91:137-168.
109. Duvic M, Talpur R, Ni X, et al. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood*. 2007;109:31-39.
110. Olsen EA, Kim YH, Kuzel TM, et al. Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol*. 2007;25:3109-3115.
111. Gore SD, Baylin S, Sugar E, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. *Cancer Res*. 2006;66:6361-6369.
112. Issa JP. DNA methylation in the treatment of hematologic malignancies. *Clin Adv Hematol Oncol*. 2005;3:684-686.
113. Issa JP. Optimizing therapy with methylation inhibitors in myelodysplastic syndromes: dose, duration, and patient selection. *Nat Clin Pract Oncol*. 2005;2(suppl 1):S24-S29.
116. Gaymes TJ, Padua RA, Pla M, et al. Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis? *Mol Cancer Res*. 2006;4:563-573.
118. Tsai HC, Li H, Van Neste L, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell*. 2012;21:430-446.
119. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, et al. Phase I/II study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. *Blood*. 2006;108:3271-3279.
120. Meng F, Sun G, Yu Y, et al. Anticancer efficacy of cisplatin and trichostatin A or 5-aza-2′-deoxycytidine on ovarian cancer. *Brit J Cancer*. 2013;108:579-586.
121. Juergens RA, Wrangle J, Vendetti FP, et al. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov*. 2011;1:598-607.
122. Fu S, Hu W, Iyer R, et al. Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. *Cancer*. 2011;117:1661-1669.
123. Matei D, Fang F, Shen C, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res*. 2012;72:2197-2205.
125. Ververis K, Hiong A, Karagiannis TC, Licciardi PV. Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. *Biologics*. 2013;7:47-60.
126. Gilbertson RJ, Graham TA. Cancer: resolving the stem-cell debate. *Nature*. 2012;488:462-463.
127. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. *Nature*. 2012;488:527-530.
128. Schepers AG, Snippert HJ, Stange DE, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science*. 2012;337:730-735.

129. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
130. Yardley DA, Ismail-Khan R, Klein P. Results of ENCORE 301, a randomized, phase II, double-blind, placebo-controlled study of exemestane with or without entinostat in postmenopausal women with locally recurrent or metastatic estrogen receptor-positive (ER+) breast cancer progressing on a non-steroidal aromatase inhibitor (AI)*ASCO Meeting Abstracts*. 2011;29(suppl 27):268.
131. Pruitt K, Zinn RL, Ohm JE, et al. Inhibition of SIRT1 reactivates silenced cancer genes without loss of promoter DNA hypermethylation. *PLoS Genet*. 2006;2:344-352.
132. Daigle SR, Olhava EJ, Therkelsen CA, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell*. 2011;20:53-65.
133. Okada Y, Feng Q, Lin Y, et al. hDOT1L links histone methylation to leukemogenesis. *Cell*. 2005;121:167-178.
134. Schwartz BE, Hofer MD, Lemieux ME, et al. Differentiation of NUT midline carcinoma by epigenomic reprogramming. *Cancer Res*. 2011;71:2686-2696.
135. Zuber J, Shi J, Wang E, et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature*. 2011.
136. Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature*. 2010;468:1067-1073.
137. Dawson MA, Prinjha RK, Dittmann A, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature*. 2011;478:529-533.
138. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell*. 2011;146:904-917.

References

1. Allis C, Jenuwein T, Reinberg D. *Epigenetics*. vol. 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2007.
2. Kornberg RD, Lorch Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell*. 1999;98:285-294.
3. Aalfs JD, Kingston RE. What does "chromatin remodeling" mean? *Trends Biochem Sci*. 2000;25:548-555.
4. Li H, Ilin S, Wang W, et al. Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. *Nature*. 2006;442:91-95.
5. Kingston R, Tamkun JW. Transcriptional regulation by Trithorax group. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:231-248.
6. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev*. 2002;16:6-21.
7. Bird AP, Wolffe AP. Methylation-induced repression—belts, braces, and chromatin. *Cell*. 1999;99:451-454.
8. Jenuwein T. The epigenetic magic of histone lysine methylation. *FEBS J*. 2006;273:3121-3135.
9. Jenuwein T. Re-SET-ting heterochromatin by histone methyltransferases. *Trends Cell Biol*. 2001;11:266-273.
10. Kouzarides T. Histone methylation in transcriptional control. *Curr Opin Genet Dev*. 2002;12:198-209.
11. Kubicek S, Schotta G, Lachner M, et al. The role of histone modifications in epigenetic transitions during normal and perturbed development. *Ernst Schering Res Found Workshop*. 2006:1-27.
12. Lachner M, Jenuwein T. The many faces of histone lysine methylation. *Curr Opin Cell Biol*. 2002;14:286-298.
13. Forneris F, Binda C, Vanoni MA, Mattevi A, Battaglioli E. Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process. *FEBS Lett*. 2005;579:2203-2207.
14. Tsukada Y, Fang J, Erdjument-Bromage H, et al. Histone demethylation by a family of JmjC domain-containing proteins. *Nature*. 2006;439:811-816.
15. Yamane K, Toumazou C, Tsukada Y, et al. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell*. 2006;125:483-495.
16. Bestor TH. The host defence function of genomic methylation patterns *Novartis Found Symp*. 1998;214:187-195. Discussion 195-189, 228-132.
17. Bird AP. CpG-rich islands and the function of DNA methylation. [Review]. *Nature*. 1986;321:209-213.
18. Brockdorff N, Turner BM. Dosage compensation in mammals. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:321-340.
19. Bartolomei MS. Epigenetics: role of germ cell imprinting. *Adv Exp Med Biol*. 2003;518:239-245.
20. Ferguson-Smith AC, Surani MA. Imprinting and the epigenetic asymmetry between parental genomes. *Science*. 2001;293:1086-1089.
21. Litt MD, Simpson M, Gaszner M, Allis CD, Felsenfeld G. Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. *Science*. 2001;293:2453-2455.
22. Yisraeli J, Frank D, Razin A, Cedar H. Effect of in vitro DNA methylation on beta-globin gene expression. *Proc Natl Acad Sci U S A*. 1988;85:4638-4642.
23. Tamaru H, Selker EU. Synthesis of signals for de novo DNA methylation in *Neurospora crassa*. *Mol Cell Biol*. 2003;23:2379-2394.
24. Jackson JP, Johnson L, Jasencakova Z, et al. Demethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma*. 2004;112:308-315.
25. Ooi SK, Qiu C, Bernstein E, et al. DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA. *Nature*. 2007;448:714-717.
26. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415-428.
27. Jones PA, Laird PW. Cancer epigenetics comes of age. *Nat Genet*. 1999;21:163-167.
28. Feinberg AP. The epigenetics of cancer etiology. *Semin Cancer Biol*. 2004;14:427-432.
29. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*. 1999;99:247-257.
30. Xu GL, Bestor TH, Bourc'his D, et al. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature*. 1999;402:187-191.
31. Berman BP, Weisenberger DJ, Aman JF, et al. Regions of focal DNA hypermethylation and long-range hypomethylation in colorectal cancer coincide with nuclear lamina-associated domains. *Nat Genet*. 2012;44:40-46.
32. Baylin SB, Jones PA. A decade of exploring the cancer epigenome—biological and translational implications. *Nat Rev Cancer*. 2011;11:726-734.
33. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
34. Baylin SB, Jones PA. Epigenetic determinants of cancer. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:457-476.
35. Esteller M. Epigenetics in cancer. *N Engl J Med*. 2008;358:1148-1159.
36. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
37. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer*. 2006;6:107-116.
38. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet*. 2002;31:141-149.
39. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer*. 2005;5:223-231.
40. Ecker JR, Bickmore WA, Barroso I, Pritchard JK, Gilad Y, Segal E. Genomics: ENCODE explained. *Nature*. 2012;489:52-55.
41. Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu Rev Genet*. 2004;38:413-443.
42. Fujimura Y, Isono K, Vidal M, et al. Distinct roles of Polycomb group gene products in transcriptionally repressed and active domains of Hoxb8. *Development*. 2006;133:2371-2381.
43. Grossniklaus U, Paro R. Transcriptional silencing by polycomb group. In: Allis CD, Jenuwein T, Reinberd D, eds. *Epigenetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006:211-230.
44. Jenuwein T, Allis CD. Translating the histone code. *Science*. 2001;293:1074-1080.
45. Galm O, Rountree MR, Bachman KE, Jair KW, Baylin SB, Herman JG. Enzymatic regional methylation assay: a novel method to quantify regional CpG methylation density. *Genome Res*. 2002;12:153-157.
46. Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet*. 2000;25:338-342.
47. Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet*. 2000;24:88-91.
48. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome—components and functional correlates. *Genes Dev*. 2006;20:3215-3231.
49. Kirmizis A, Bartley SM, Farnham PJ. Identification of the polycomb group protein SU(Z)12 as a potential molecular target for human cancer therapy. *Mol Cancer Ther*. 2003;2:113-121.
50. Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A*. 2003;100:11606-11611.
51. Varambally S, Dhanasekaran SM, Zhou M, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*. 2002;419:624-629.
52. Fraga MF, Ballestar E, Villar-Garea A, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet*. 2005;37:391-400.
53. McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB. Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. *Cancer Res*. 2006;66:3541-3549.
54. De Carvalho DD, Sharma S, You JS, et al. DNA methylation screening identifies driver epigenetic events of cancer cell survival. *Cancer Cell*. 2012;21:655-667.
55. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. *J Mol Cell Biol*. 2009;1:82-92.
56. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med*. 2009;360:765-773.

57. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med*. 2009;361:1058-1066.
58. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature*. 2012;483:474-478.
59. Sasaki H, Setoguchi T, Matsunoshita Y, Gao H, Hirotsu M, Komiya S. The knock-down of overexpressed EZH2 and BMI-1 does not prevent osteosarcoma growth. *Oncol Rep*. 2010;23:677-684.
60. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553-567.
61. Noushmehr H, Weisenberger DJ, Diefes K, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*. 2010;17:510-522.
62. Widschwendter M, Fiegl H, Egle D, et al. Epigenetic stem cell signature in cancer. *Nat Genet*. 2007;39:157-158.
63. Schlesinger Y, Straussman R, Keshet I, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet*. 2006.
64. Ohm JE, McGarvey KM, Yu X, et al. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat Genet*. 2007;39:237-242.
65. Easwaran H, Johnstone SE, Van Neste L, et al. A DNA hypermethylation module for the stem/progenitor cell signature of cancer. *Genome Res*. 2012.
66. Baylin SB. Stem cells, cancer, and epigenetics. *Stem Book*. 2009:2-14.
67. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet*. 2006;7:21-33.
68. O'Hagan HM, Wang W, Sen S, et al. Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG Islands. *Cancer Cell*. 2011;20:606-619.
69. Sherr CJ. Cancer cell cycles. *Science*. 1996;274:1672-1677.
70. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16(INK4a). *Nature*. 2006.
71. Krishnamurthy J, Ramsey MR, Ligon KL, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature*. 2006;443:453-457.
72. Molofsky AV, Slutsky SG, Joseph NM, et al. Increasing p16(INK4a) expression decreases forebrain progenitors and neurogenesis during ageing. *Nature*. 2006;443:421-426.
73. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci U S A*. 1998;95:11891-11896.
74. Herman JG, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res*. 1995;55:4525-4530.
75. Wong DJ, Paulson TG, Prevo LJ, et al. p16(INK4a) lesions are common, early abnormalities that undergo clonal expansion in Barrett's metaplastic epithelium. *Cancer Res*. 2001;61:8284-8289.
76. Holst CR, Nuovo GJ, Esteller M, et al. Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. *Cancer Res*. 2003;63:1596-1601.
77. Foster SA, Wong DJ, Barrett MT, Galloway DA. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol Cell Biol*. 1998;18:1793-1801.
78. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature*. 1998;396:84-88.
79. Reynolds PA, Sigaroudinia M, Zardo G, et al. Tumor suppressor P16INK4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells. *J Biol Chem*. 2006;281:24790-24802.
80. Hammerman PS, Hayes DN, Wilkerson MD, et al. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012;489:519-525.
81. Jen J, Harper JW, Bigner SH, et al. Deletion of p16 and p15 genes in brain tumors. *Cancer Res*. 1994;54:6353-6358.
82. Siu IM, Robinson DR, Schwartz S, et al. The identification of monoclonality in human aberrant crypt foci. *Cancer Res*. 1999;59:63-66.
83. Kinzler KW, Vogelstein B. Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*. 1997;386:761-763.
84. Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science*. 1997;275:1784-1787.
85. Suzuki H, Watkins DN, Jair KW, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet*. 2004;36:417-422.
86. Rattner A, Hsieh JC, Smallwood PM, et al. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci U S A*. 1997;94:2859-2863.
87. Seligson DB, Horvath S, Shi T, et al. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature*. 2005;435:1262-1266.
88. Cao R, Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell*. 2004;15:57-67.
89. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003;349:2042-2054.
90. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer*. 2004;4:707-717.
91. Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer*. 2003;3:253-266.
92. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res*. 2001;61:3225-3229.
93. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A*. 1996;93:9821-9826.
94. Bailey VJ, Easwaran H, Zhang Y, et al. MS-qFRET: a quantum dot-based method for analysis of DNA methylation. *Genome Res*. 2009;19:1455-1461.
95. Li M, Chen WD, Papadopoulos N, et al. Sensitive digital quantification of DNA methylation in clinical samples. *Nat Biotechnol*. 2009;27:858-863.
96. Belinsky SA, Liechty KC, Gentry FD, et al. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer Res*. 2006;66:3338-3344.
97. Machida EO, Brock MV, Hooker CM, et al. Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. *Cancer Res*. 2006;66:6210-6218.
98. Gu J, Berman D, Lu C, et al. Aberrant promoter methylation profile and association with survival in patients with non-small cell lung cancer. *Clin Cancer Res*. 2006;12:7329-7338.
99. Brock MV, Hooker CM, Ota-Machida E, et al. DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med*. 2008;358:1118-1128.
100. Glockner SC, Dhir M, Yi JM, et al. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res*. 2009;69:4691-4699.
101. Zou H, Allawi H, Cao X, et al. Quantification of methylated markers with a multiplex methylation-specific technology. *Clin Chem*. 2012;58:375-383.
102. Jeronimo C, Usadel H, Henrique R, et al. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. *J Natl Cancer Inst*. 2001;93:1747-1752.
103. Issa JP. CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol*. 2000;249:101-118.
104. Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med*. 2000;343:1350-1354.
105. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352:997-1003.
106. Yoo CB, Jones PA. Epigenetic therapy of cancer: past, present and future. *Nat Rev Drug Discov*. 2006;5:37-50.
107. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004;429:457-463.
108. Marks PA, Richon VM, Miller T, Kelly WK. Histone deacetylase inhibitors. *Adv Cancer Res*. 2004;91:137-168.
109. Duvic M, Talpur R, Ni X, et al. Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood*. 2007;109:31-39.
110. Olsen EA, Kim YH, Kuzel TM, et al. Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin Oncol*. 2007;25:3109-3115.
111. Gore SD, Baylin S, Sugar E, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. *Cancer Res*. 2006;66:6361-6369.
112. Issa JP. DNA methylation in the treatment of hematologic malignancies. *Clin Adv Hematol Oncol*. 2005;3:684-686.

113. Issa JP. Optimizing therapy with methylation inhibitors in myelodysplastic syndromes: dose, duration, and patient selection. *Nat Clin Pract Oncol*. 2005;2(suppl 1):S24-S29.
114. Kornblith AB, Herndon 2nd JE, Silverman LR, et al. Impact of azacytidine on the quality of life of patients with myelodysplastic syndrome treated in a randomized phase III trial: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2002;20:2441-2452.
115. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. *J Clin Oncol*. 2002;20:2429-2440.
116. Gaymes TJ, Padua RA, Pla M, et al. Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis? *Mol Cancer Res*. 2006;4:563-573.
117. Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R. Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc Natl Acad Sci U S A*. 1997;94:4681-4685.
118. Tsai HC, Li H, Van Neste L, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell*. 2012;21:430-446.
119. Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, et al. Phase I/II study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. *Blood*. 2006;108:3271-3279.
120. Meng F, Sun G, Zhong M, et al. Anticancer efficacy of cisplatin and trichostatin A or 5-aza-2′-deoxycytidine on ovarian cancer. *Brit J Cancer*. 2013;108:579-586.
121. Juergens RA, Wrangle J, Vendetti FP, et al. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov*. 2011;1:598-607.
122. Fu S, Hu W, Iyer R, et al. Phase 1b-2a study to reverse platinum resistance through use of a hypomethylating agent, azacitidine, in patients with platinum-resistant or platinum-refractory epithelial ovarian cancer. *Cancer*. 2011;117:1661-1669.
123. Matei D, Fang F, Shen C, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res*. 2012;72:2197-2205.
124. Liang G, Chan MF, Tomigahara Y, et al. Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol Cell Biol*. 2002;22:480-491.
125. Ververis K, Hiong A, Karagiannis TC, Licciardi PV. Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. *Biologics*. 2013;7:47-60.
126. Gilbertson RJ, Graham TA. Cancer: resolving the stem-cell debate. *Nature*. 2012;488:462-463.
127. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. *Nature*. 2012;488:527-530.
128. Schepers AG, Snippert HJ, Stange DE, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science*. 2012;337:730-735.
129. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
130. Yardley DA, Ismail-Khan R, Klein P. Results of ENCORE 301, a randomized, phase II, double-blind, placebo-controlled study of exemestane with or without entinostat in postmenopausal women with locally recurrent or metastatic estrogen receptor-positive (ER+) breast cancer progressing on a non-steroidal aromatase inhibitor (AI)*ASCO Meeting Abstracts*. 2011;29(suppl 27):268 2011.
131. Pruitt K, Zinn RL, Ohm JE, et al. Inhibition of SIRT1 reactivates silenced cancer genes without loss of promoter DNA hypermethylation. *PLoS Genet*. 2006;2:344-352.
132. Daigle SR, Olhava EJ, Therkelsen CA, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell*. 2011;20:53-65.
133. Okada Y, Feng Q, Lin Y, et al. hDOT1L links histone methylation to leukemogenesis. *Cell*. 2005;121:167-178.
134. Schwartz BE, Hofer MD, Lemieux ME, et al. Differentiation of NUT midline carcinoma by epigenomic reprogramming. *Cancer Res*. 2011;71:2686-2696.
135. Zuber J, Shi J, Wang E, et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature*. 2011.
136. Filippakopoulos P, Qi J, Picaud S, et al. Selective inhibition of BET bromodomains. *Nature*. 2010;468:1067-1073.
137. Dawson MA, Prinjha RK, Dittmann A, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature*. 2011;478:529-533.
138. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell*. 2011;146:904-917.

Peter M. Howley

6

Infectious Agents and Cancer

Overview of Cancer and Infectious Agents

Infectious agents are second only to tobacco use as a potentially preventable cause of cancer in humans. Estimates vary between 15% and 30% as to the percentage of cancers worldwide that are associated with an infectious etiology.[1,2] The burden is greater in the developing world, but the impact even in the United States and other developed countries is significant. Specific viruses, parasites, and bacteria are now associated with specific human cancers. These are discussed in some detail in this chapter.

There are three major mechanisms by which an infectious agent can cause a cancer, and these may involve the initiation as well as the promotion of carcinogenesis.[3] The first is perhaps the most common, resulting from the infectious agent causing a persistent infection with chronic inflammation. This can result in the formation of reactive oxygen and nitrogen species by macrophages at the site of the infection. These reactive molecules can damage DNA and proteins as well as membranes and thus contribute to carcinogenesis.[4] Chronic inflammation due to the persistent infection can then lead to repeated cycles of cell damage and cellular proliferation. Cells that are cycling in the presence of reactive molecules are more likely to acquire genetic mutations that could contribute to the initiation as well as the promotion of cancer. A second mechanism involves the direct participation of the infectious agent in the transformation of the cell through the activation of a cellular oncogene pathway or the inactivation of a tumor suppressor gene. A third mechanism, relevant to the human immunodeficiency virus (HIV), is that the infection may result in immunosuppression and the decreased recognition of infected or transformed cells by the host immune system. Indeed, many of the cancers observed in immunosuppressed patients, such as those infected with HIV, are those that have been associated with other viruses.

The recognition of an infectious etiology for specific cancers provides the opportunities to prevent those cancers by preventing or controlling the infections. Depending on the infectious agent, this could involve public health measures or changes in cultural practices. It could also involve the development of vaccines to prevent the initial infections, as has now been achieved for hepatitis B virus (HBV) and the genital-tract human papillomaviruses (HPVs). It could also involve the treatment of the infections with specific therapeutics or the development of novel therapies for those agents for which there are not yet specific or effective drugs.

Viruses and Cancer

History of Viral Oncology

Viral oncology has its beginnings as a discipline from observations made during the early part of the 20th century: in 1908, when the transmissibility of avian leukemia was first described by Ellermann in Denmark, and in 1911, when the transmissibility of an avian sarcoma in chickens was described by Rous.[5,6] The importance of these findings was not appreciated at the time, and the full impact on virology and medicine was not recognized until the 1950s. Indeed, the work of Peyton Rous[6] showing that cell-free extracts containing a filterable agent from a sarcoma in chickens could induce tumors in injected chickens within a few weeks was finally recognized with a Nobel Prize in 1966. Rous's original work pointed out that a filterable agent (the working definition of a virus at that time) not only was capable of inducing tumors, but also was responsible for determining the phenotypic characteristics of the tumor. Because these studies were carried out in birds and not in mammals, however, this early work was consigned to the rank of avian curiosities.

In the 1930s, Richard Shope published a series of papers demonstrating the cell-free transmission of tumors in rabbits. The first studies involved fibromatous tumors found in the footpads of wild cottontail rabbits that could be transmitted by injecting cell-free extracts in either wild or domestic rabbits; a virus referred to as the Shope fibroma

virus is now known to be a pox virus. Other studies carried out by Shope demonstrated that cutaneous papillomatosis in wild cottontail rabbits could also be transmitted by cell-free extracts. In a number of cases, these benign papillomas would progress spontaneously into squamous cell carcinomas in infected domestic rabbits or in the infected cottontail rabbits.[7,8] In general, however, the field of viral oncology lay dormant until the early 1950s, with the discovery of the murine leukemia viruses by Ludwig Gross[9] and of the mouse polyomavirus by Gross, Stewart, and Eddy.[10,11] The identification of tumor viruses in mice opened the field of experimental viral oncology. Researchers had the hope that these initial observations in mammals could be extended to humans and that a fair proportion of human tumors might also be found to have a viral etiology. The Special Viral Cancer Program in the 1960s at the National Cancer Institute grew from this intense interest in viral oncology and the belief that human tumor viruses would be identified.

Many of the most important developments in modern molecular biology derive from studies in viral oncology from the 1960s and 1970s. The discovery of reverse transcriptase, the development of recombinant DNA technology, the discovery of messenger RNA splicing, and the discovery of oncogenes and, more recently, tumor suppressor genes all have been developments that emerged directly from studies in viral oncology. Oncogenes were first recognized as cellular genes that had been acquired by retroviruses through recombination processes to convert them into acute transforming RNA tumor viruses. It is now recognized that oncogenes participate in many different types of tumors and can be involved at different stages of tumorigenesis and viral oncology. This has contributed significantly to our concepts in nonviral carcinogenesis. It is likely that the direct-transforming, oncogene-transducing retroviruses do not play a major causative role in naturally occurring cancers in animals or in humans, but rather represent laboratory-generated recombinants. A list of human viruses that are now associated with human cancer is presented in Table 6-1. Also included on this list are viruses such as the transforming adenoviruses that, although capable of transforming normal cells into malignant cells in the laboratory, have not been associated with any known human tumors.

Also listed in Table 6-1 are cofactors that are believed to be important in the carcinogenic processes associated with each of these viruses. It is clear that none of these viruses by themselves is sufficient for the induction of the specific neoplasias with which they have been associated. Each of the viruses associated with these human cancers is thought to be involved at an early step in carcinogenesis. Subsequent cellular genetic events such as somatic mutations are thought to

Table 6-1 Human Viruses with Oncogenic Properties

Virus Family	Type	Human Tumor	Cofactors	Comments
Adenovirus	Types 2, 5, 12	None	N/A	Important experimental model
Hepadnavirus	Hepatitis B virus (HBV)	Hepatocellular carcinoma (HCC)	Aflatoxin, alcohol, smoking	Causative
Herpesvirus	Epstein-Barr virus (EBV)	Burkitt's lymphoma	Malaria	EBV
		EBV-associated malignancies in immunosuppressed individuals	Immunodeficiency	Causative
		Nasopharyngeal carcinoma	Nitrosamines, genetic	Causative
		Hodgkin's lymphoma	?	Variable association
		Gastric carcinoma	?	Variable association
	KSHV (HSV8)	Kaposi's sarcoma	AIDS	Causative
		Castleman's disease	?	Causative
		Primary effusion lymphomas	?	Causative
Flavivirus	Hepatitis C virus (HCV)	Hepatocellular carcinoma	Aflatoxin	Causative
Papillomaviruses	HPV16, -18, -31, -33, -35, -39, and others	Anogenital cancers, some upper airway cancers	Smoking, oral contraceptives, ?other factors	Causative
	HPV5, -8, -17, -20, -47	Skin cancer	Genetic disorder (EV), UV, immunosuppression	Unclear if causative
Polyomavirus	MCV	Merkel cell cancer	UV, immunosuppression	Likely causative
	BK	?Prostate preneoplastic lesions	?	Unclear if causative
	JC	?Brain tumors	?	Unclear if causative
	SV40*	?Mesotheliomas, brain tumors, etc.	?	Unlikely
Retroviruses	HTLV-1	ATL	?Genetic	Causative
	HTLV-2	None	N/A	Not associated with human malignancy

ATL, Adult T-cell lymphoma; *N/A*, not applicable; *SV40*, simian virus 40. AIDS, Acquired Immunodeficiency Syndrome; HPV, human papillomavirus; MCV, Merkel cell virus; BK, BK human polyomavirus; JC, JC human polyomavirus; HTLV, human T-cell leukemia virus; UV, ultraviolet irradiation.
*SV40 is simian virus 40, a nonhuman primate virus closely related to the human polyomaviruses BK and JC.

be important at the subsequent steps involved in the multistep process of malignant progression.

Human Papillomaviruses

The human papillomaviruses cause warts and papillomas and are associated with some specific human cancers. The papillomaviruses have been found exclusively in higher vertebrates, in species ranging from birds to man. More than 140 different types of HPVs are now recognized, and new types are still being recognized. Because serologic reagents are not available for all types, some HPVs have been typed by their DNA sequence. Many of the HPVs have now been fully or partially sequenced, and these DNA sequence data now lead their phylogenetic organization (Figure 6-1).[12] Some of these viruses as well as the clinical syndromes with which they are associated are presented in Table 6-2.

Virus-Host Interactions

The papillomaviruses have a specific tropism for squamous epithelial cells (keratinocytes). The functions of the

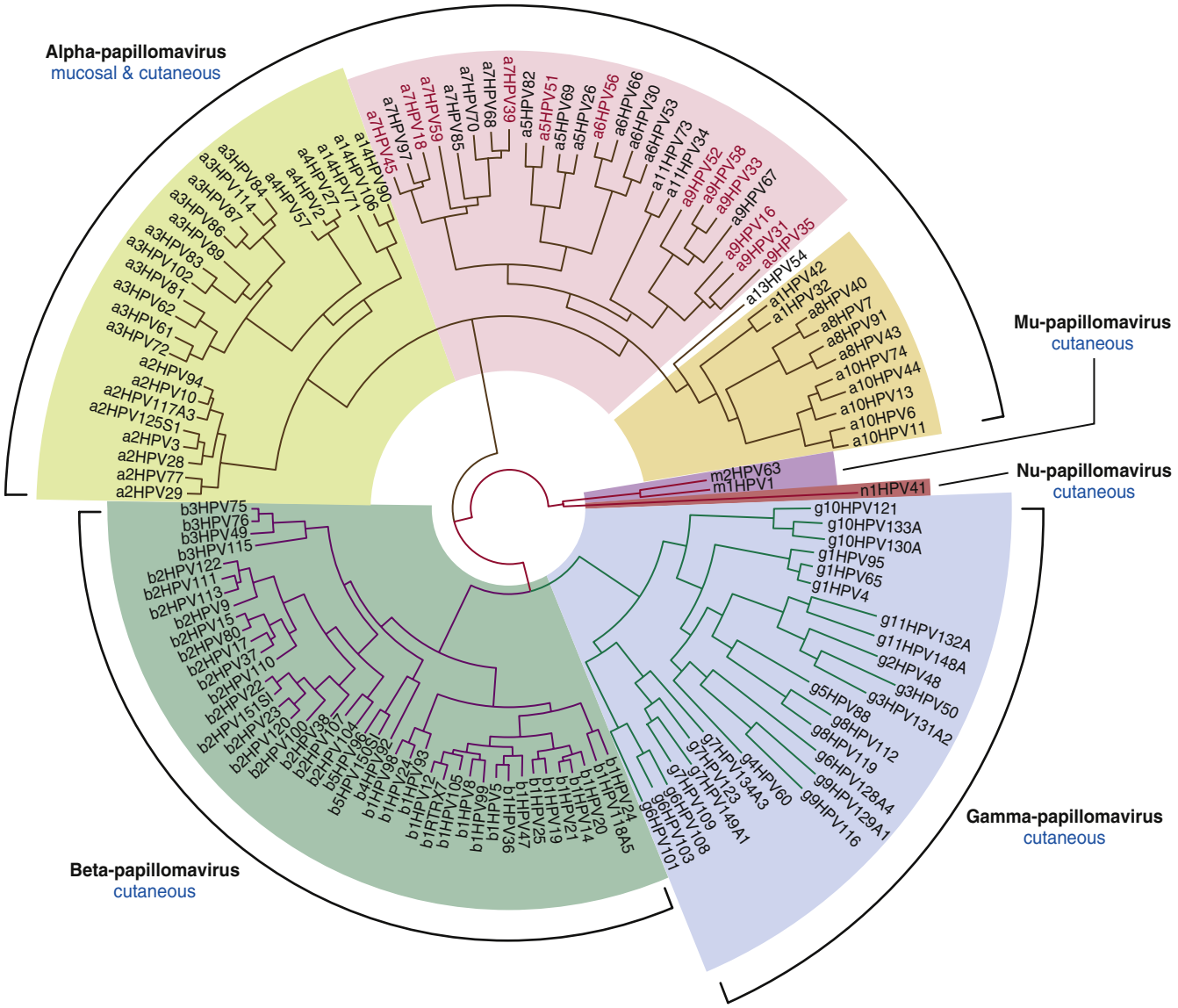


Figure 6-1 Phylogenetic tree demonstrating the evolutionary relationship among HPVs HPVs comprise five groups with different epithelial tropisms and disease associations. The alpha-papillomaviruses include the low-risk mucosal types (many of which are within the tan-shaded branch) that cause genital warts, and the high-risk mucosal types (contained within the branch highlighted with pink shading) associated with anogenital preneoplasias and cancers. Although the cutaneous HPV types (most of which are contained within the light green-[alpha], green-[beta], and blue-[gamma] shaded branches) are not generally associated with cancers, certain beta types have been implicated in the development of nonmelanoma skin cancers (NMSCs) in immunosuppressed individuals and in epidermodysplasia verruciformis (EV) patients. The lowercase letter and number preceding the HPV type refer to its genus and species. (*Reprinted with permission from Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses.* Vaccine. *2012;30 Suppl 5:55-70).*

Table 6-2 Association of HPVs and Clinical Lesions

A. Cutaneous Lesions and HPVs		
Clinical association viral types		
Plantar wart	HPV1	
Common wart	HPV2, 4	
Mosaic wart	HPV2	
Multiple flat warts	HPV3, 10, 28, 41	
Macular plaques in EV	HPV5, 8, and other beta HPV types	
Butcher's warts	HPV7	
B. Genital Tract HPVs		
Condyloma acuminata (exophytic)	HPV6, 11	
Giant condyloma (Bushke-Lowenstein tumor)	HPV6, 11	
Subclinical infection	All genital tract HPV types	
Squamous intraepithelial lesions	HPV16, 18, 31, 33, etc.	
Bowenoid papulosis	HPV16, 18, etc.	
Cervical cancer	Strong association, "high risk"	HPV16, 18, 31, 45
	Moderate association	HPV33, 35, 39, 51, 52, 56, 58, 59, 68
	Weak or no association, "low risk"	HPV6, 11, 26, 42, 43, 44, 51, 53, 54, 55, 66
Other anogenital cancers (vulvar, penile, etc.)	HPV16 and other "high-risk" HPV types	
Respiratory papillomas	HPV6, 11	
Conjunctival papillomas	HPV6, 11	
Focal epithelial hyperplasia (oral cavity)	HPV13, 32	
Oropharyngeal cancer	HPV16	

EV, Epidermodysplasia verruciformis; *HPV*, human papillomavirus.

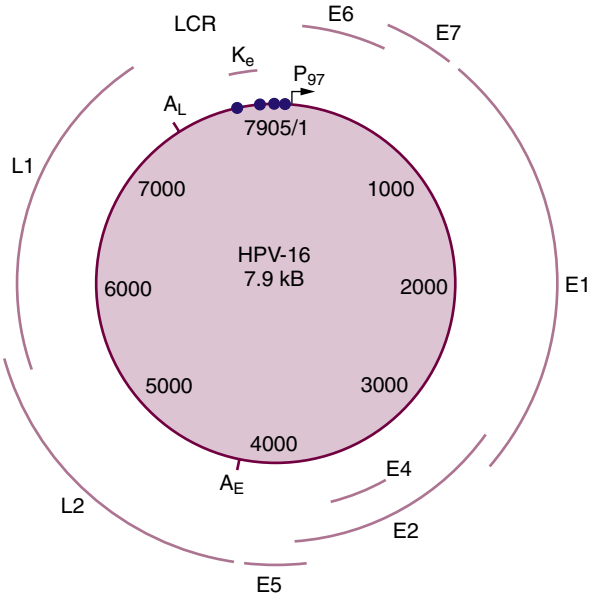


Figure 6-2 Map of the HPV16 genome The nucleotide numbers are noted within the circular maps, transcription proceeds clockwise, and the major open reading frames (E1 to E7, L1, and L2) are indicated. The transcriptional promoter that directs the expression of E6 and E7 is designated P_{97}. A_E and A_L represent the polyadenylation signals for the early and late transcripts, respectively. The viral long control region *(LCR)* contains the viral transcriptional and replication regulatory elements. The closed circles on the genome represent the four E2 binding sites that have been noted in the LCR.

papillomaviruses necessary for the production of infectious virions, which include vegetative viral DNA replication and the synthesis of the capsid proteins, occur only in the fully differentiated squamous epithelial cells of a papilloma. Viral capsid protein synthesis and virion assembly occur only in the terminally differentiated cells of the upper layers of the epithelium. The viral genome is present in the epithelial cells of all layers of the epithelium, including the basal layer. It is generally believed that the expression of specific viral genes in the basal layer and in the lower layers of the epidermis stimulates cellular proliferation and alters the keratinocyte differentiation profile, characteristic of a wart. As squamous epithelial cells migrate upward through the layers and differentiate, the pattern of viral gene expression changes, resulting in the expression of the late genes (L1 and L2) that encode the capsid proteins.

The genomic organization of all the papillomaviruses is quite similar. All of the open reading frames (ORFs) that could serve to encode proteins for these viruses are located on only one of the two viral DNA strands, and only one strand is transcribed. The HPV genome can be divided into three distinct regions: (1) an "early" region that encodes the viral proteins (E1, E2, etc.) involved in viral DNA replication, transcriptional regulation, and cellular transformation, (2) a "late" region that encodes the viral major (L1) and minor (L2) capsid proteins, and (3) a region called the "long control region" (LCR) or alternatively, the upstream regulatory region (URR) that does not contain any ORFs, but does contain cis-regulatory elements, including the origin of DNA replication and important transcription factor binding sites. A diagram of the organization of the HPV16 genome, which is typical of all the HPVs, is shown in Figure 6-2.

The late genes (L1 and L2) are expressed only in the more differentiated cells of the epithelium, whereas the early (E) region genes are expressed throughout the epithelium. A more detailed description of the biology and molecular biology of the papillomaviruses can be found in *Fields Virology*.[13]

Papillomaviruses and Cancer

Only some papillomaviruses are associated with cancer. These include several animal PVs as well as a subset of the HPVs. From an experimental standpoint, the cottontail

Table 6-3 Papillomaviruses Associated with Cancers in Their Natural Host

Species	Cancers	Viruses	Other Factors
Human	Anogenital cancers	HPV16, -18, -31, etc.	Tobacco
	Oropharyngeal cancers	HPV16	
	Malignant progression of respiratory papillomas	HPV6, -11	X-irradiation, smoking
	Nonmelanoma skin cancer	HPV5, -8, -17, and other beta genus HPVs	Genetic (EV), UV light, and immunosuppression
Rabbit	Skin cancer	CRPV	Methylcholanthrene and coal tar (experimental)
Cattle	Alimentary tract cancers	BPV-4	Bracken fern
	Conjunctival cancers	Not characterized	UV light
Sheep	Skin cancer	Not characterized	UV light

BPV, Bovine papillomavirus; *CRPV,* cottontail rabbit papillomavirus; *EV,* epidermodysplasia verruciformis; *HPV,* human papillomavirus; UV, ultraviolet.

rabbit papillomavirus (CRPV) that was first identified by Richard Shope has been extensively studied as a model for papillomavirus-induced carcinogenesis.[7] One of the principal features of carcinogenic progression associated with PVs is the synergy often observed between a specific virus and other carcinogenic factors (Table 6-3). In the case of CRPV, carcinomas develop at an increased frequency in papillomas that are painted with coal tar or with methylcholanthrene.[14,15] These CRPV-associated carcinomas contain viral DNA that is transcriptionally active, and the carcinogenic properties are believed to map to specific viral genes. There are additional instances where animal papillomaviruses have been associated with naturally occurring cancers, including the bovine papillomavirus type 4 (BPV-4) that causes esophageal papillomatosis and is associated with squamous cell carcinomas of upper alimentary tract.[16] Major interest today, however, is in the role of specific HPVs with the human cancers with which they have been associated.

HPV and Cervical Cancer

Cervical cancer is the third most common malignancy among women worldwide, with approximately 530,000 newly diagnosed cases each year and about 275,000 deaths annually.[2,17] About 80% of cervical cancer occurs in developing countries, where it is frequently the most common cancer of women, accounting for as many as one quarter of female cancers. It occurs less frequently in developed countries. In the United States, there are about 12,000 newly diagnosed cases annually, and about one third of these women will die of their malignant disease. The incidence of cervical cancer in the United States varies considerably among ethnic and socioeconomic groups, with the rate among African American women being about twice that of White women.[18]

Most cervical cancers develop in the transformation zone, the region of the cervix where the columnar cells of the endocervix adjoin with the stratified squamous epithelium of the exocervix. About 85% of cervical cancers are squamous cell cancers, the remainder being adenocarcinomas and small-cell neuroendocrine tumors. The progression of normal cervical epithelial cells to malignant squamous cell carcinomas typically occurs through a series of dysplastic changes over a time span of many years, a process that is the basis of the Pap smear screening program. The histologic classifications of cervical intraepithelial neoplasia (CIN) grades 1, 2, and 3 correspond, respectively, to mild dysplasia, moderate dysplasia, and severe dysplasia or carcinoma in situ. Because of the long interval for the progression of cervical dysplasia to invasive cancer, Pap smear screening programs can identify the vast majority of premalignant lesions for appropriate treatment, thereby preventing the development of most cases of cervical cancer in countries with screening programs. Most CIN lesions do not progress to cancer but resolve; the lesser the degree of dysplasia, the more likely the lesion is to resolve.

Cervical cancer had been recognized for decades as linked to a sexually transmitted agent, long before sexually transmitted HPV infection was implicated in its pathogenesis. Venereal transmission of a carcinogenic factor with a long latency had been suggested by the early epidemiologic studies. Sexual promiscuity, an early age of onset of sexual activity, and poor sexual hygiene conditions were identified by these studies as risk factors in women for cervical carcinoma. The counterpart to cervical cancer in the male is penile cancer, because there is a correlation between the incidence rates of these two cancers in different geographic areas. Compelling evidence linking an HPV infection with cervical carcinoma followed the observation that some of the morphologic changes characteristic of cervical dysplasia seen on Pap smears were due to a papillomavirus infection.[19] The cell with its characteristic perinuclear clearing and abnormally shaped nucleus that is diagnostic for a cervical papillomavirus infection is the koilocyte. The presence of papillomavirus particles, papillomavirus-specific capsid antigens, and HPV DNA within the cervical preneoplastic lesions provides confirmation of the HPV etiology of cervical dysplasia.

Harald zur Hausen and his colleagues identified the first papillomavirus DNAs, HPV16 and HPV18, in cervical cancer tissues in the 1980s.[20,21] Using HPV DNAs as probes under conditions of reduced hybridization stringency, most cervical carcinomas were shown to harbor these or related HPV DNAs. Subsequent studies led to the identification of approximately 40 different HPVs, mostly from the alpha genus, associated with genital tract lesions, a subset of which are associated with human cervical cancer.

In addition to HPV16 and HPV18, which account for approximately 70% of human cervical cancers, HPV types 31, 33, 35, 45, 52 and 58 account for a total of 95% of HPV-positive cancers.[22] DNAs from these same HPV types are found in other human genital carcinomas, including penile carcinomas, some vulvar carcinomas, and some perianal carcinomas, as well as in the precancerous intraepithelial lesions of each of theses sites (PIN, VIN, and PAIN).

The genital-tract–associated HPVs have been classified as either high risk or low risk based on whether the lesions with which they are associated are at significant risk for malignant progression. The low-risk viruses such as HPV6 and HPV11 are associated with venereal warts, lesions that only rarely progress to cancer. The high-risk viruses such as HPV16 and HPV18 are associated with CIN and cervical cancer. The other high-risk viruses include HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68, and HPV82. Virtually all cases of CIN3 and cervical cancer contain a high-risk HPV DNA.[13] HPV-positive cervical cancers and cell lines derived from HPV-positive cervical cancer tissues often, but not always, contain integrated viral DNA. In those cancers in which the viral DNA is integrated, the pattern of integration is clonal, indicating that the integration event preceded the clonal outgrowth of the tumor. Integration of the viral DNA does not occur at specific sites in the host chromosome, although in some cancers the HPV DNA has integrated in the vicinity of known oncogenes. For instance, in the HeLa cell line (which is an HPV18-positive cervical carcinoma cell line), integration of the HPV18 genome is within approximately 50 kilobases of the c-myc locus on human chromosome 8. It is possible that such an integration event might provide a selective growth advantage to the cell and thus might contribute to neoplastic progression.

The Role of HPV in Cervical Cancer

In cervical cancers, only a subset of the viral genes is expressed, and there is no production of virus by the cancer cells. The integration of the viral genome appears to play an important role leading to the deregulated expression of the viral E6 and E7 genes.[13] The E6 and E7 genes are invariably expressed in HPV-positive cervical cancers. Integration of the HPV genome into the host chromosome in the cancers often results in the disruption of the viral E1 or E2 genes. Because HPV E2 is a viral regulatory factor that negatively regulates expression of the E6 and E7 genes, the disruption of E2 results in the derepression of the E6/E7 promoter, leading to deregulated expression of E6 and E7. Indeed, the introduction of E2 into cervical cancer cell lines results in the induction of cellular senescence by repressing E6 and E7 expression.

The E6 and E7 genes of the high-risk genital-tract–associated HPVs function as oncogenes. Expression of E6 and E7 together is sufficient for the efficient immortalization of primary human cells, most notably primary human keratinocytes, the normal host cell for the human papillomaviruses.[23] In contrast to the immortalization properties of the HPV16 and HPV18 E6 and E7 proteins, the E6 and E7 proteins encoded by the low-risk viruses are either inactive or only weakly active in the same assays.

The major cellular targets for E6 and E7 are the tumor suppressor proteins p53 and pRB, respectively. E6 and E7 are, however, polyfunctional proteins and have many other biochemical activities and biologic properties that may be relevant to their activities in cervical carcinogenesis.[13] A common theme among the small DNA tumor viruses (i.e., the polyomaviruses, the adenoviruses, and the cancer-associated HPVs) is that the immortalization and transformation properties of their encoded oncoproteins are in part due to their interactions with critical cellular regulatory proteins (Figure 6-3). The HPV E7 proteins share some amino acid sequence similarity to adenovirus E1A and with portions of the SV40 large T antigen, in regions that are critical for the transformation activities of these oncoproteins. These regions of amino acid sequence similarity shared by these viral oncoproteins specify the binding to the product of the retinoblastoma tumor suppressor gene, pRB, and the related pocket proteins p107 and p130. Studies have established that a major component of the transformation activities of these viral oncoproteins is due to their respective abilities to complex and functionally inactivate pRB and the related pocket proteins. The binding of these viral oncoproteins to pRB, p107, and p130 leads to cellular proliferation rough the activation of genes under the control of the E2F family of transcription factors. The transcriptional activities of the E2F family of transcription factors are modulated by

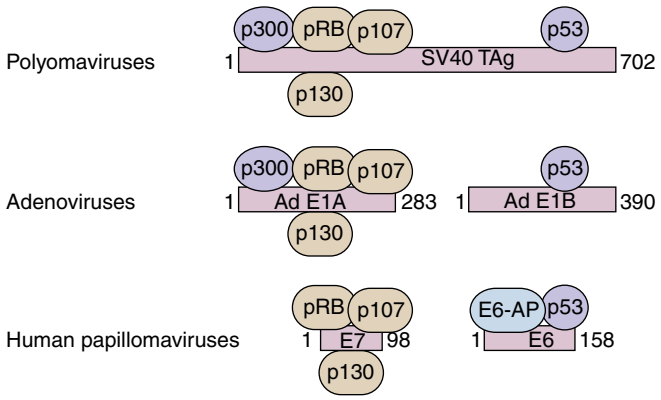


Figure 6-3 The transforming proteins encoded by three distinct groups of DNA tumor viruses target similar cellular proteins The binding of HPV E6 oncoproteins to p53 is mediated by the cellular ubiquitin ligase E6AP (also known as UBE3A) that is hijacked by E6 to target the ubiquitylation of p53.

pRB and the other pocket proteins. When complexed with E2F proteins, they act as transcriptional repressors, and when dissociated from the pocket proteins by E7, E1A, or SV40 T-antigen, the E2F proteins function to activate transcription of their target genes. In the normal life cycle of the papillomaviruses, the binding of E7 to pRB is essential for the activation of the cell-cycle DNA replication machinery in differentiated keratinocytes that had otherwise exited the cell cycle. The small DNA tumor viruses, including HPV, depend on the host cell DNA replication machinery for the replication of their viral genomes. Because this machinery is only expressed in the S phase of the cell cycle, these viruses must stimulate cellular proliferation and drive the cell into the S phase in order to replicate the viral genomes. In the case of HPV, this occurs through E7 binding pRB, freeing up the E2F family of transcription factors.

A number of genetic studies indicate that E7 binding to pRB and its related pocket proteins is not sufficient to account for its immortalization and transforming functions, indicating that there are additional cellular targets and activities of E7 that are relevant to cellular transformation. Indeed, a large number of putative cellular targets for E7 have been identified using a variety of biochemical approaches and proteomic approaches. The physiologic relevance of many of these interactions is not yet clear,[24] but some of these targets appear to be relevant to cancer. For instance, E7 can interact with cyclin-dependent kinase inhibitors. Like Ad E1A, HPV16 E7 interacts with and abrogates the inhibitory activity of p21(cip1) and p27(kip1) and thus has effects on cell cycle progression and keratinocyte differentiation. In addition, high-risk HPV E7 can cause genomic instability in normal human cells.[25] HPV16 E7 induces G1/S and mitotic cell cycle checkpoint defects and uncouples synthesis of centrosomes from the cell division cycle.[26] This causes formation of abnormal multipolar mitoses, leading to chromosome missegregation and aneuploidy.[27] Moreover, there is an increased incidence of double-strand DNA breaks and anaphase bridges, suggesting that in addition to numerical abnormalities, high-risk E7 proteins also induce structural chromosome aberrations.[28] Abnormal centrosome duplication rapidly results in genomic instability and aneuploidy, one of the hallmarks of a cancer cell. This activity is therefore likely to be functionally relevant to the contribution of high-risk HPVs to malignant progression.

The immortalization/transformation properties of the E6 protein were first revealed by studies using primary human genital squamous epithelial cells.[29,30] Efficient immortalization of primary human cells by HPV16 or HPV18 requires both the E6 and E7 genes. Like SV40 large T antigen and the 55-kDa protein encoded by adenovirus E1B, the E6 proteins of the high-risk HPVs can complex with p53.[31] The interaction of E6 with p53 is not direct but is mediated by a cellular protein, called the *E6-a*ssociated *p*rotein (E6AP).[32] E6AP is a ubiquitin protein ligase and, in the presence of E6, directly participates in the ubiquitylation of p53.[33] Multi-ubiquitylated p53 is then recognized and degraded by the 26S proteasome. Consequently, the half-life and level of p53 are low in E6-immortalized cell lines and in HPV-positive cancers. Through its ubiquitylation of p53, HPV 16 E6 can abrogate the transcriptional activation and repression properties of p53 and disrupt the ability of wt p53 to mediate cell-cycle arrest in response to DNA damage. The p53 protein can sense DNA damage and prevent the replication of mutated DNA through its transcriptional activation of the p21 cyclin-dependent kinase inhibitor. Thus, the functional abrogation of p53 by high-risk HPV E6 results in decreased genomic stability and accumulation of DNA abnormalities in high-risk HPV E6–expressing cells. Hence, E6 can be directly implicated in the establishment and propagation of genomic instability, a hallmark in the pathology of malignant progression of cervical lesions.

The development of centrosome abnormalities and aneuploidy, two important related pathologic processes, appears to be initiated before viral DNA integration and may contribute to this process.[34] High-risk HPV can induce abnormal centrosome duplication, which can result in genomic instability and aneuploidy.[27] The deregulation of this mitotic event appears to depend on both E6 and E7, with the latter protein being most responsible for the effect. Indeed, the deregulated viral oncogene expression may result in chromosomal instability and aneuploidy, enhancing the likelihood of viral DNA integration.

A number of additional cellular targets have now been identified for the high-risk E6 proteins in an attempt to define additional p53-independent cellular targets. The reader is referred to the current edition of *Fields Virology*[13] for a more comprehensive discussion of these additional activities, some of which may be relevant to the role of E6 in cervical carcinogenesis. Two activities are of particular importance, however, and are discussed here. The first is the binding to cellular PDZ domain–containing proteins. Interestingly, the high-risk E6 oncoproteins contain an X-(S/T)-X-(V/I/L)-COOH motif at the extreme C terminus that can mediate the binding to cellular PDZ domain–containing proteins. This motif is unique in the high-risk HPV E6 proteins and is not present in the E6 proteins of the low-risk HPV types. E6 serves as a molecular bridge between these PDZ domain proteins and E6AP, facilitating their ubiquitylation and mediating their proteolysis. Among the PDZ domain proteins implicated as E6 targets are hDlg, the human homologue of the *Drosophila melanogaster* Discs large tumor suppressor, and hScrib, the human homologue of the *Drosophila* Scribble tumor suppressor.[35,36] Additional PDZ domain proteins have also been shown to be capable of

binding to E6. Several PDZ-containing proteins have been shown to be involved in negatively regulating cellular proliferation. Therefore, some of the p53-independent transforming activities of the high-risk E6 oncoproteins may be linked to their ability to bind and degrade some of these PDZ motif–containing proteins.

A second important p53-independent activity of HPV16 E6 is its ability to activate telomerase in keratinocytes through the transcriptional upregulation of the rate-limiting catalytic subunit of human telomerase (hTERT).[37,38] Maintenance of telomere length is an important step in cancer and can occur through the transcriptional activation of hTERT expression or through the activation of the ALT recombination pathway. Activation of hTERT is observed in most human cancers, including HPV-positive cervical cancers. The mechanism by which E6 activates the hTERT promoter has not been yet fully elucidated but could involve the direct activation of a cellular transcription factor by E6 or perhaps the E6AP-dependent degradation of a negative regulator of the hTERT promoter.

Infection by a high-risk HPV does not always cause cancer. Indeed, cancer is a rare outcome of an HPV infection, even for HPV16 and HPV18. Expression of the E6 and E7 oncogenes is not sufficient for malignant progression. The time period between infection by a high-risk HPV and the development of invasive cancer can be several decades. Thus, infection with a high-risk HPV constitutes only the initial step in cervical carcinogenesis; the genetic information carried by the virus per se is not sufficient to cause cancer. Epidemiologic studies have suggested that smoking is a risk factor for developing cervical carcinoma.[39] The recognition that other factors are involved in the progression to cervical carcinomas suggests that papillomavirus infections may work synergistically with these other factors.

Tumor progression is, however, a complex process that involves multiple additional genetic loci. Specific chromosomal abnormalities have been detected in cervical cancer, including the loss of heterozygosity on the short arm of chromosome 3 (3p).[40] This locus contains the FHIT (fragile histidine triad) gene,[41] and its expression is inversely correlated with the severity of the lesion and prognosis.[42] In addition, loss of 11q23 may involve the tumor suppressor of lung cancer gene (TSLC1), which is implicated in cell adhesion.[43] An additional possibility is that cellular mutations or epigenetic changes could be involved in downregulating HLA antigen class I alleles and the ability of an HPV-positive cancer cell to be recognized by the host cellular immune response.

HPV and Other Cancers

The high-risk genital tract HPV types can infect other genital areas that contain stratified squamous epithelium and cause intraepithelial neoplasias and cancer. HPV DNA, usually HPV16, can be found in a subset of cancers of the vulva, vagina, and penis.[44,45] Giant condyloma acuminata, also called the Buschke-Lowenstein tumor, is a low-grade, locally invasive squamous cell carcinoma that involves the external genitalia and is associated with low-risk HPV types, usually 6 or 11.[46,47]

Anal cancer is also associated with high-risk HPV infection, and the rate of anal HPV infection appears to be similar to that of cervical infection, although anal HPV infection has been studied less systematically than cervical infection.[48] As with cervical cancer, high-risk HPV can be found in most anal cancers, usually HPV16; most anal cancers arise in the transition zone between columnar and squamous epithelium. The risk of anal cancer in the general population appears to be much lower than for cervical cancer, and the incidence of anal cancer in women is less than one tenth that of cervical cancer. The risk of anal cancer among individuals who are human immunodeficiency virus (HIV)-positive is much greater than in the general population, with especially high rates for HIV-positive male homosexuals.[49]

HPV is linked to some head and neck cancers, although not to the majority of the cancers in this region. HPV16 accounts for about 90% of the HPV-positive tumors. Most of these HPV-associated cancers are located in the oropharynx, which includes the tonsils, tonsillar fossa, base of the tongue, and soft palate. It is not understood why the HPV-positive tumors preferentially develop in the oropharynx. In the United Sates, the incidence of these oropharyngeal cancers, which usually develop at a younger age than the HPV-negative cancers, increased more than threefold between 1988 and 2004.[50] Genital-oral sex may be a risk factor for these tumors, and the risk of HPV infection and cigarette smoking may be more than additive. The HPV-positive tumors tend to have a characteristic basaloid pathology and share many molecular features with those of HPV-positive anogenital tumors. The tumors usually have integrated HPV DNA expressing E6 and E7. Their p53 and pRB genes are wild type, and the vast majority of them expresses p16,[51] in contrast to the HPV-negative tumors, which tend to have mutant p53 and to be p16-negative. There is thus far, however, no clearly identifiable premalignant oropharyngeal lesion for HPV-positive tumors. HPV-positive oropharyngeal cancers carry a better prognosis than the HPV-negative ones.[52,53]

Esophageal carcinomas in humans have also been reported to have some association with HPVs; however, the data as yet are not as convincing as they are with the anogenital cancers and with oropharyngeal cancers. The esophagus is lined by a squamous epithelium, and squamous cell papillomas of the esophagus have been described in humans.

Additional studies seem warranted to investigate a possible role of HPV in human esophageal cancers. There have also been sporadic reports associating occasional human tumors, including colon cancer, ovarian cancer, prostate cancer, and even melanomas, with the presence of HPV DNA in the literature. In general it seems prudent to be skeptical of such reports until systematic and well-carried-out studies are confirmed in multiple laboratories.

HPV and Epidermodysplasia Verruciformis

EV is a very rare disorder in which affected individuals have a unique susceptibility to cutaneous HPV infection.[54] The warts usually develop in childhood, become widespread, do not tend to regress, and in approximately 30% of patients may progress to squamous cell cancers. Several types of lesions may occur in the same patient. Some lesions are typical flat warts (usually caused by HPV3 or HPV10), whereas others are flat, scaly, red-brown macules. The scaly lesions are associated with a range of beta-HPV types, most frequently HPV5 and HPV8. Patients with EV are often infected by multiple HPV types.

In approximately one half of affected patients, EV occurs as an inherited disorder. Inheritance appears to have an autosomal recessive pattern in most affected families, although one family with apparent X-linked recessive inheritance has been reported. Cases with autosomal recessive inheritance appear to be genetically heterogeneous, because the condition in different families has been mapped to two distinct chromosomal loci,[55] and two adjacent novel genes (EVER1 and EVER2) have now been molecularly identified at one of these loci (17q25).[56]

EV patients do not have an increased susceptibility to clinical infection with other microbial agents, including other HPVs. In addition, the EV-specific HPV types have now been found in normal skin of many individuals, so the EV patients are unusual in that these HPV types produce clinically apparent lesions. However, clinical lesions associated with EV-specific HPV types have been described in other immunosuppressed individuals, such as renal transplant patients.[57] Patients with EV often have impaired cell-mediated immunity, which is believed to be important with regard to the manner in which they respond to infections by this subset of cutaneous HPVs.

About one third of EV patients develop skin cancers in association with their lesions. Most of the malignant tumors remain local, but regional and distant metastases may occur. The risk of malignant progression is limited to the pityriasis-like lesions, which are the lesions that contain the beta-HPV types. HPV5 and HPV8 appear to be the most oncogenic, because most of the skin cancers contain one of these two types. The EV carcinomas usually arise in sun-exposed areas, suggesting that ultraviolet radiation may play a co-carcinogenic role with the specific HPVs in the etiology of these cancers. Mutations in the p53 gene are common in EV-associated cancer,[58] in contrast to the mucosal cancers associated with HPV. Studies have also established that the viral genomes are transcriptionally active within these carcinomas.[59]

HPV and Nonmelanoma Skin Cancer

Nonmelanomas skin cancers (NMSCs) are subdivided into basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). They generally arise on exposed areas, and UV exposure is a predominant risk factor. Immunosuppressed individuals are at high risk for developing warts as well as premalignant lesions and NMSC, especially SCC, in sun-exposed areas.[60,61] The consistent finding of certain beta genus HPV types in SCC associated with EV and other immunosuppressed individuals makes HPV infection an attractive etiologic agent for at least some NMSCs in individuals who do not have EV.[62] Beta HPVs encode potential viral oncoproteins that could interfere with UV-induced apoptosis,[63] which might allow keratinocytes with UV-induced mutations to survive and progress to carcinomas. Although beta HPV DNA can be frequently detected in SCC using sensitive PCR-based detection methods, it is also frequently detected in normal skin.[64-66] The genome copy number is usually much less than one copy per tumor cell.[67] A recent study employing an unbiased analysis involving high-throughput sequencing of randomly primed mRNAs detected virtually no HPV transcripts in SCC specimens.[68] Overall, the association between HPV infection and NMSC is therefore considered weak at present, because expression of predominant HPV types has not been as clearly identified in NMSC as it is in EV-associated skin cancers or in mucosal cancers associated with the high-risk alpha genus HPV types. It is formally possible that the beta HPV types have a role in the initiation of NMSC but that they are not required for cancer maintenance.[67]

HPV Preventive Vaccine

A major advance in the prevention of human cancer has been the development of an effective preventive vaccine for the major genital tract HPVs. The vaccine is a subunit vaccine consisting of the major capsid protein (L1) that can self-assemble into virus-like particles (VLPs), which are empty capsids that closely resemble authentic virions morphologically and immunologically.[69] The L1 VLPs are highly immunogenic, inducing high titers of neutralizing antibodies that are conformationally dependent and type specific. Two commercial prophylactic HPV vaccines have been developed and approved by the FDA. GlaxoSmithKline's Cervarix is a bivalent vaccine composed of L1 VLPs of HPV16 and 18, whereas Merck's Gardasil is a quadrivalent

vaccine composed of L1 VLPs of HPV6, 11, 16, and 18. Both vaccines are generally safe, able to induce high titers of capsid-reactive antibodies, and highly effective at preventing acquisition of cervical infection and low- and high-grade CIN caused by the types targeted by the vaccine.[70] Both vaccines also induce a modest degree of protection against cervical infection caused by specific nonvaccine types closely related to HPV16 or 18. For instance, both vaccines induced partial protection against persistent infection by HPV31. Cervarix, but not Gardasil, induced significant protection against HPV45, and neither vaccine protected significantly against HPV35 or 58.[71-73] One can anticipate that second-generation VLP vaccines may be able to protect against an even higher proportion of HPV infection by incorporating VLPs from a larger number of HPV types. Although 70% of cervical cancers are caused by HPV16 or HPV18, 30% are caused by the other high-risk HPV types.

Although Cervarix and Gardasil have now been licensed in more than 100 countries, they have been introduced into the national vaccination programs of only about 30 countries, mostly the most developed ones. National programs have been centered on vaccination of preadolescent or adolescent girls, ages 9 to 15 years, because more than 90% of HPV-associated cancer worldwide occurs in women.[74] However, recent evidence indicates that Gardasil protects young men from genital warts and anal cancer precursors, providing a rationale for considering male vaccination programs. Furthermore, the increase in HPV-positive head and neck oropharyngeal cancer also suggests a rationale for male vaccination programs.

There are several important unresolved issues for the current VLP vaccines.[69] For instance, the VLP vaccine is expensive and is not heat stable, two characteristics that might impede its use in developing countries where the cervical cancer disease burden is greatest. Because of the type specificity, the current vaccines are unlikely to protect against a substantial proportion of other high-risk HPV-type infections, so it will be important for vaccinated women to continue to undergo cervical cancer screening. Additional approaches to improve the vaccine seem warranted. Merck has indicated that a nonavalent (nine HPV targets) VLP vaccine is currently in clinical trials.[75] In addition, the use of L2 represents a potential alternative approach to developing a prophylactic vaccine against a broader spectrum of HPV types. Although they are not as immunogenic as the L1 neutralization epitopes, at least some of the L2 neutralization epitopes induce cross-neutralizing antibodies against papillomaviruses from different types.[76,77] In addition, modifications of the L1 capsid protein allow the self-assembly of capsomeres that are highly immunoprotective, can be produced in bacteria, and are more stable.[78]

Epstein-Barr Virus

Epstein-Barr virus (EBV) is a common virus with a worldwide distribution. More than 90% of individuals worldwide have been infected by the time they reach adulthood. EBV was discovered through studies of a lymphoma described in young children in certain parts of East Africa. Although this childhood lymphoma had been previously recognized, it was first clearly defined as a unique entity with characteristic clinical, pathologic, and epidemiologic features by Dennis Burkitt in 1958.[79,80] His early descriptive studies speculated that the lymphoma could be due to a virus because its geographic distribution in a belt across equatorial Africa was similar to that of yellow fever. In 1964, Epstein and Barr described virus particles of the herpesvirus family in lymphoblastoid cells from patients with Burkitt's lymphoma (BL).[81,82] The finding of such virus particles in lymphoid lines, however, was not restricted to tissues from BL patients; these particles could also be observed in cell lines established from patients with other malignancies, from patients with infectious mononucleosis, and even occasionally from normal individuals. Nonetheless, EBV was the first virus to be recognized as a human tumor virus.

Virus-Host Cell Interactions

EBV is a double-stranded DNA virus and is a member of the herpesvirus family. Other members of the human herpesvirus family include herpes simplex viruses types 1 and 2, varicella zoster virus, cytomegalovirus, human herpesvirus types 6 and 7, and Kaposi's sarcoma herpesvirus (KSHV, also known as HSV8). The mature EBV particle is essentially indistinguishable from those of the other herpesviruses. Herpesviruses are large viruses, measuring 150 to 180 nm in diameter, and contain a large double-stranded DNA genome of about 170,000 base pairs. In addition to this central core of genetic material, the virus particle consists of a capsid layer made up of capsomeres in an icosahedral shape and an outer lipoprotein envelope. EBV is considered a member of the gamma herpesviruses because of its tropism for lymphoid cells, both in vivo and in vitro. EBV infects epithelial cells of the oropharynx and B-lymphocytes. The infection of B cells is a latent infection in which there is no replication of the virus and the cells are not killed. EBV proteins are serologically distinct from proteins of other human herpesviruses.

Antibodies to EBV are prevalent in all human populations, and high titers of antibody correlate with infectious mononucleosis.[83] Primary infection in adolescents in developed countries causes infectious mononucleosis. EBV also causes X-linked lymphoproliferative disease type 1 (XLP-1), an often fatal disease, in patients with a mutation in the *SAP* (SLAM-associated protein) gene.[84] High titers of EBV are

also often seen in patients with specific malignancies: BL, nasopharyngeal carcinoma (NPC), and Hodgkin's lymphoma, as discussed later.[84] EBV is associated with B-cell lymphomas in immunosuppressed individuals, particularly those with HIV or organ transplant recipients.

EBV has several distinct programs of gene expression in infected cells, a lytic cycle and a latent cycle. The lytic cycle results from the phased expression of viral proteins that ultimately results in the replication of the virus and the production of infectious virions. The replicative cycle of EBV does not inevitably result in the lysis of the infected host cell, because EBV virions are produced by budding from the infected cell. Latent infections do not result in the production of progeny virions. In latently infected B lymphocytes, the genome circularizes an episome in the cell nucleus. In B-lymphoid cells that harbor and express the EBV genome in a latent state, there is expression of a distinct subset of viral proteins, including the EBV-induced nuclear antigens (EBNAs): EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-leader protein (EBNA-LP). In addition, EBV encodes two latent-infection–associated membrane proteins (LMPs), and two small nonpolyadenylated RNAs (EBERs) that are also expressed in EBV latently infected cells. Molecular genetic analyses using specifically mutated EBV recombinants have revealed that EBNA-3B, LMP2, the EBERs, and most of the viral genome that is expressed in lytic infection can be mutated without a significant effect on the ability of the virus to transform primary B lymphocytes.[84] The other EBNAs and LMP1 are important for lymphocyte transformation. LMP2 is important in maintaining latency by preventing lytic infection in response to lymphocyte activation signals. A detailed description of the molecular biology of EBV and its normal biology is provided in the recent edition of *Fields Virology*.[84]

Two of these genes, EBNA-2 and LMP1, are particularly important with regard to viral latency and EBV immortalization of human B cells. EBNA-1 is a DNA binding protein that binds to an EBV origin of DNA replication called oriP and mediates genome replication and partitioning during division of the latently infected cells. EBNA-1 also possesses a glycine-alanine repeat that functions to impair antigen processing and MHC class I–restricted antigen presentation of EBNA-1, thereby inhibiting the CD8-restricted cytotoxic T-cell recognition of virus-infected cells.

LMP1 has been shown to alter the effect of the growth properties of rodent cells, epithelial cells, and B lymphocytes. LMP-1 is a transmembrane protein that is essential for EBV-mediated growth transformation. LMP-1 mediates signaling through the tumor necrosis factor-alpha/CD40 pathway. When expressed in normal resting B lymphocytes or in EBV-negative lymphoblastoid cell lines, LMP1 induces most B lymphocyte activation and adhesion markers, activates NFκB, and induces Bcl2 and A20, proteins important in preventing apoptosis. The C-terminal LMP1 cytoplasmic domain interacts with cellular proteins that transduce signals from the tumor necrosis factor receptor (TNFR) family. TNF signaling is critical in normal lymphoid development, and the B-lymphocyte TNFR family member, CD40, is remarkably similar in its growth-promoting and NFκB-activating effects to LMP1. The evidence supports a model that LMP1 mimics a constitutively activated TNFR.[84]

EBNA-2 is the main viral transcriptional transactivator that has effects both on viral and cellular genes. Viral proteins whose expression can be increased by EBNA-2 include the latent membrane protein (LMP1) and another membrane protein that is expressed in latently infected cells called terminal protein. EBNA-2 lacks DNA sequence-specific binding activity and is dependent on interactions with sequence-specific cell proteins for the recognition of enhancer elements. EBNA-2 binds the cellular RBPJκ protein and is recruited to promoters regulated by RBPJκ, including the Notch pathway. EBNA-3A and -3C also regulate transcription in lymphocyte transformation and, like EBNA-2, EBNA-3A and -3C also achieve specificity in their interaction with viral and cellular promoters by interacting with the cell protein RBPJκ. Through the interactions of EBNA-2, EBNA-3A, and EBNA-3C with the cell protein JK, EBV therefore affects the cellular Notch signaling pathway.[84]

Burkitt's Lymphoma

Endemic BL that occurs in Africa and New Guinea in the malaria belt is an EBV-positive malignancy that occurs several years after the primary infection with EBV. BL is a monoclonal lymphoma, as opposed to infectious mononucleosis, which is a polyclonal disease caused by EBV. African BL is clinically characterized by rapid growth of the tumor at nonlymphoid sites such as the jaw or the retroperitoneum. The tumor is of B-cell origin and is morphologically similar to the small noncleaved cells of normal lymphoid follicles.[85] The proliferation of the B lymphocytes and the reduction in virus-specific cytotoxic T cells associated with malaria is believed to result in an increased EBV viral load, enhancing the risk of the c-myc translocations characteristic of BL.[86] The failure of the T-cell immune response to control this proliferation might be an early step providing the enhanced opportunity for further mutation, oncogenic transformation, and lymphomagenesis in the actively dividing B-cell population. In contrast, only 15% to 20% of the non-African, sporadic BL tumors are EBV positive. BLs regularly contain chromosomal abnormalities, often in regions that contain the immunoglobulin genes, most notably chromosomes 2, 14, and 22. In more than 90% of BL, a translocation of the long arm of chromosome 14 (containing the heavy chain immunoglobulin genes) to chromosome

8 (containing the *c-myc* oncogene) is observed.[87] Less frequent translocations involve chromosome 2 (kappa light chain) and chromosome 22 (lambda light chain).[88] The translocations with chromosomes 2 and 22 generally involve reciprocal translocations to the distal arm of chromosome 8, containing *c-myc*. The expression of the *c-myc* oncogene following this translocation is deregulated because of the proximity of the *c-myc* oncogene to the transcriptional control elements of the immunoglobulin genes that are active in B cells. Overexpression of the *c-myc* oncogene itself is not sufficient for malignant transformation of a B cell. Additional mutations can then occur in these B cells, leading eventually to the emergence of a monoclonal B-cell neoplasm. Thus EBV does not act directly as an oncogene, but rather indirectly as a polyclonal B-cell mitogen, setting the stage for the translocation to activate the *c-myc* oncogene and other mutations.

What is the role of specific EBV genes in the maintenance of BL? As noted earlier, EBNA-2 and LMP1 appear to be the mediators of EBV-induced growth effects in B lymphocytes. These, however, are not expressed in BL and are therefore not required for BL growth. It is possible that altered *myc* expression may replace the need for EBV oncogenic functions. Furthermore, the down-modulation of the EBV EBNA and LMP functions may actually be advantageous to tumor development, allowing the cell to escape from T-cell–mediated immune surveillance. It has been shown that the EBNAs and LMP can serve as targets of immune cytotoxic T cells, and that LMP-1 induction of cell adhesion molecules can enhance the HLA-restricted killing of EBV-infected T cells.[89]

Nasopharyngeal Carcinoma

NPC is also linked to EBV. NPC occurs in adults and, in general, males outnumber females 2 to 1. Although worldwide the annual incidence rates are low, there are areas in China (especially the southern provinces) where the rate of disease can be 50 cases per 100,000 per year in men over 50. Because the incidence among individuals of Chinese descent remains high, irrespective of where they live, a genetic susceptibility has been proposed. Environmental factors have been implicated as risk factors for NPC, including fumes, chemicals, smoke, and ingestion of salt-cured fish that contain nitrosamines. The rate of NPC in the United States and Europe is between 0.5 and 2 per 100,000.

EBV genomes are found in nearly all biopsies of anaplastic NPC specimens from all over the world.[90,91] The genome is present in the epithelial cells of the tumors (but not in the infiltrating lymphocytes), and it is noteworthy that all forms of NPC contain clonal EBV episomes, indicating that the tumors arise from a single infected cell.[92] The EBV genome is transcriptionally active within these tumors, and the regions that are transcribed in the NPC biopsies are the same as those expressed in latently infected lymphocytes.[93] These molecular observations are consistent with an active role for EBV in the neoplastic processes involved in NPC. Patients with NPC have elevated levels of IgG antibodies to EBV capsid and early antigens. NPC patients have serum IgA antibodies to capsid and early antigen, likely reflecting the local production of such antibodies in the nasopharynx. Cytogenetic studies on NPC xenografts have identified abnormal markers on a number of different chromosomes. Loss of heterozygosity has been noted by studies using restriction fragment length polymorphisms (RFLPs) on two different regions of chromosome 3 mapping to 3p25 and 3q14 in a very high percentage of NPC specimens.

The presence of immunoglobulin markers for EBV (IgA/VCA and IgA/EA) has provided the opportunity for early serologic identification of patients with NPC. The frequency of IgA antibody to the EBV capsid antigen of 150,000 Chinese studied was found to be 1%. About 20% of the patients with elevated IgA antibodies to VCA had NPC, however, when biopsied. Thus, early detection using serologic tests can be applied in areas where NPC is prevalent, possibly leading to early therapeutic intervention.

EBV-Associated Malignancies in Immunocompromised Individuals

Strong evidence for the oncogenic potential for EBV comes from its association with a variety of malignancies in immunocompromised individuals. These include EBV-positive lymphoproliferative disease in children with primary immunodeficiencies affecting T-cell competence (such as those with the Wiskott-Aldrich syndrome), posttransplant lymphoproliferative disease (PTLD), B-cell lymphomas in patients with acquired immunodeficiency syndrome (AIDS), and smooth muscle cell tumors of the immunocompromised patient.[84]

EBV is associated with B-cell lymphomas in patients with acquired or congenital immunodeficiencies and in organ transplant recipients. These lymphomas can be distinguished from the classical BLs in that the tumors are often polyclonal. Also, the tumors do not demonstrate the characteristic chromosomal abnormalities of BL described earlier. The pathogenesis of these lymphomas involves a deficiency in the affector mechanisms needed to control EBV-transformed cells.

The association between EBV and leiomyomas and leiomyosarcomas in immunocompromised patients was unexpected and has now been seen in the context of acquired immunodeficiency and in organ transplant patients. From

the EBV standpoint, the pathogenesis and role of EBV are not yet well understood.[84]

Hodgkin's Lymphoma

Serologic and epidemiologic studies suggesting a possible link between Hodgkin's lymphoma (HL) and EBV were supported by molecular studies that demonstrated EBV DNA, RNA, and proteins in HL pathologic specimens.[94-97] The four histologic types of HD vary in the rate of their EBV positivity: 80% to 90% for lymphocyte depleted, 60% to 75% for mixed cellularity, 20% to 40% for nodular sclerosis, and 10% for lymphocyte rich.[84] EBV proteins and DNA can be demonstrated in approximately 40% of Reed-Sternberg cells. Furthermore, expression of LMP1 in the Reed-Sternberg and Hodgkin's cells can be demonstrated in a large percentage of the EBV-positive cases of MC and NS subtypes, although EBNA-2 expression is not detected.[96,98] Activation of the NFκB pathway is a common feature of HD, and the activation of this pathway by LMP1 to promote lymphocyte proliferation and survival may suggest a role for EBV in promoting HD. The evidence that EBV, when present, plays a causative role in the pathogenesis of some cases of HD is considered strong.[84]

Gastric Carcinoma

EBV is present in about 10% of gastric cancers worldwide, with a similar prevalence in the United States and Asia. As in NPC, it is the epithelial cells, not infiltrating lymphocytes, that contain the EBV DNA, and the DNA is clonal or oligoclonal, consistent with a role for EBV in tumor development. The EBV genes expressed in the cancers include EBNA-1 and the nonpolyadenylated viral RNAs known as the EBERs, as well as several other genes including the latent membrane protein 2A (LMP2A).[84]

Non-Hodgkin's Lymphomas and Other Malignancies in Nonimmunocompromised Individuals

EBV is present in approximately 6% of non-Hodgkin's lymphoma (NHL) cases, including some B-cell lymphoma, peripheral T-cell lymphoma, angioblastic T-cell lymphoma, lymphomatoid granulomatosis, pyothorax-associated lymphoma, extranodal NK/T-cell lymphoma (nasal type), aggressive NK-cell leukemia/lymphoma, and inflammatory pseudotumor-like follicular dendritic cell sarcoma.[84,99] Although there have also been reports of EBV associated with breast cancer, there is not yet compelling evidence associating the virus with the disease.[84]

Kaposi's Sarcoma Herpesvirus

The Kaposi's sarcoma–associated herpesvirus (KSHV), also known as human herpesvirus 8 (HSV8), is a gamma-2 herpesvirus. Chang, Moore, and colleagues discovered this virus in 1994 by representational difference analysis of an AIDS Kaposi's sarcoma (KS) skin lesion.[100] Since its discovery, KSHV has been linked with several other different tumors in addition to KS, namely, body-cavity–based or primary effusion lymphomas (PEL) and some plasma cell forms of multicentric Castleman's disease (MCD).

KS was initially described as an aggressive tumor by Moritz Kaposi in the 19th century. Before the onset of the AIDS epidemic, KS had been described as a rare and indolent tumor of elderly Mediterranean men and was later recognized to occur more frequently in parts of Africa. KS had also been observed among immunosuppressed organ transplant patients. KS is the most common neoplasm associated with AIDS.[101,102] The histology of KS in all of these clinical settings is similar. KS lesions contain multiple cell types, including spindle cells, which are believed to arise from an endothelial cell precursor, and infiltrating mononuclear cells. KS lesions are histologically characterized by slitlike vascular channels that give the lesions their distinctive reddish clinical appearance.

Research with PEL cell lines and on the KSHV itself quickly moved the field along and established an etiologic role for the virus in KS.[103] The identification of KSHV-positive PEL cell lines[104,105] led to the development of some initial serologic assays for epidemiologic and virologic studies of the agent. KSHV is a member of the rhadinovirus (or gamma-2) subfamily of the herpesviruses. It is the only known human rhadinovirus and is closely related to the herpesvirus saimiri of squirrel monkeys. Humans are the only known host for KSHV. Unlike other human herpesviruses, infection by KSHV is not ubiquitous, and only a small percentage of humans in developed countries are serologically positive for the virus. Infection by KSHV is characterized by a prolonged viral and clinical latency that, as with other herpesviruses, may be lifelong. In a setting of immunosuppression or immunodeficiency, individuals infected with KSHV may then develop KS or other KSHV-associated tumors, years after the primary infection. At this point, a role for KSHV has been established for KS and for PEL and MCD.

Despite the progress in the epidemiology and the molecular biology associated with KSHV, our understanding of the virology and the mechanisms of pathogenesis and carcinogenesis associated with this virus is still at an early stage. The virus has been difficult to culture in the laboratory, and much of our knowledge has been discerned from the analysis of the primary sequence by studying individually encoded genes. One characteristic of the rhadinovirus subfamily of the herpesviruses is the presence of recognizable variants of cellular genes that appear to have been captured into the viral genomes, a process that has been termed "molecular piracy." These genes are believed to play important regulatory roles

in the virus life cycle, in evading the cell's host defenses and in causing its associated pathology in the host.[106] Among the KSHV regulatory genes are viral genes that resemble cellular cytokines, cellular chemokines, the cellular interferon regulatory factor (IRF-1), the cellular apoptosis factor (FLIP), a viral homologue of Bcl-2, a viral cyclin that is resistant to inhibition by cdk inhibitors, and a chemokine receptor, among others. In addition, many of the KSHV regulatory genes resemble EBV genes or target cellular pathways that are also targeted by other DNA tumor viruses, particularly EBV. Included among this group of genes is LAMP, which is similar to the EBV LMP1 and LMP2A genes. Much of the effort in the field has been focused on these individual genes and their properties. It is beyond the scope of this chapter to go into detail about the molecular biology of KSHV and these particular studies. Instead, I refer the reader to the comprehensive chapter on the molecular biology of KSHV in the recent edition of *Fields Virology*.[107]

Viruses and Liver Cancer

Hepatocellular carcinoma (HCC) is one of the world's commonest malignancies. In China alone, there are between 500,000 and 1 million cases of HCC per year. HCC is etiologically linked to infections by two different types of viruses, hepatitis B virus (HBV) and hepatitis C virus (HCV). Though relatively rare in the West, HCC is quite prevalent in Southeast Asia and in sub-Saharan Africa. In the 1970s, this distribution was recognized to mirror the distribution of chronic HBV infection. Indeed, the long-recognized association between HCC and chronic hepatitis led to the strong presumption that chronic HBV infection predisposes to hepatic cancer. This presumption was validated in large prospective epidemiologic studies in Taiwan, in which chronic infection with HBV leading to cirrhosis was shown to be of major importance in the etiology of this tumor.[108] Chronic HBV infection was found to be associated with about a 19-fold increase of HCC mortality risk in men and a 33.5-fold increase in women.[109] The World Health Organization has estimated that 80% of HCC worldwide occurs in individuals who are chronically infected by HBV. For the remaining 20% of HCC not associated with HBV, there are a number of additional risk factors, including chronic hepatitis associated with HCV. Between 30% and 70% of HBV-negative cases of HCC are seropositive for HCV. In the United States, it has been estimated that as many as 40% of cases of HCC are due to HCV.

Hepatitis B Virus (Virus-Host Interactions)

HBV is a member of a group of the hepadnaviruses (for hepatotropic DNA viruses). HBV is the only human member of this group of viruses. Other members of this group include the woodchuck hepatitis virus (WHV), the Beechey ground squirrel hepatitis virus (GSHV), the Pekin duck hepatitis B virus (DHBV), and the grey heron hepatitis virus. These viruses share a similar structure, and each is hepatotropic, leading to persistent viral infections of the liver. The animal hepatitis viruses have been very important contributors to our understanding of the molecular biology of these viruses. Of the hepadnaviruses, only HBV and WHV have been associated with chronic active hepatitis and HCC.

The reader is referred to the *Fields Virology* chapter on the molecular biology of the hepadnaviruses for details on the virus and aspects of virus/host cell interactions.[110] Hepatitis B viral particles contain small, circular DNA molecules that are only partially double stranded. The DNA consists of a long strand with a constant length of 3220 bases and a shorter strand that varies in length from 1700 to 2800 bases in different molecules. A map of the HBV DNA genome is shown in Figure 6-4. The virion particles contain a DNA polymerase activity that is capable of repairing the single-stranded DNA region to make two fully double-stranded molecules, each approximately 3220 bases in length. For this reaction, DNA synthesis initiates at the 3′ end of the short strand that, as noted earlier, is heterogeneous among different DNA molecules. DNA synthesis terminates at the uniquely located 5′ end of the short strand when it is reached. The long strand is not a closed molecule

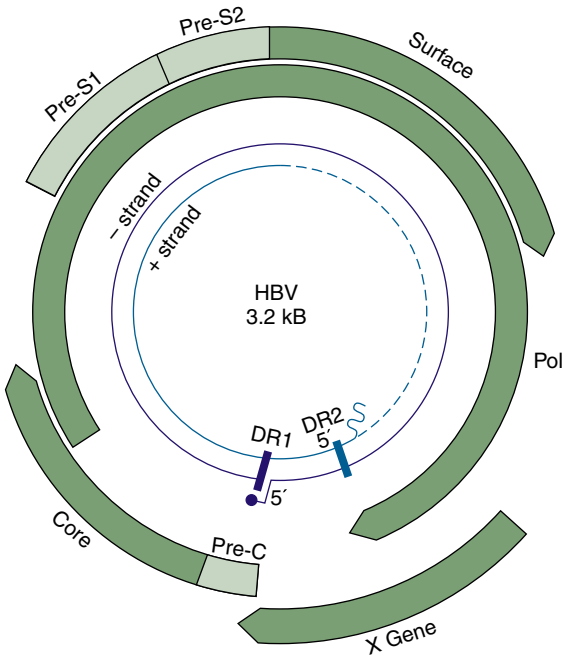


Figure 6-4 Map of the HBV genome The arrows surrounding the genome represent the four large open reading frames of the L⁻ strand with the genes they encode indicated. The broken line is the S⁺ DNA strand. The positions of the 5′ ends of the DNA strands are indicated. The locations of the direct repeats (DR1 and DR2) involved in the initiation of DNA replication are also indicated.

but contains a nick at a unique site approximately 300 base pairs from the 5′ end of the short strand.

The HBV genome has four ORFs and encodes four genes. These ORFs are designated as S and pre-S, C, P, and X. S and pre-S represent two contiguous reading frames and code for the viral surface glycoproteins. C contains the coding sequences for the core structural protein of the nucleocapsid. The P gene encodes the viral polymerase that contains reverse transcriptase activity. The X ORF encodes a basic polypeptide that has transcriptional transactivation properties that can upregulate the activity of hepadnavirus promoters. The overall structure of the genomes of all of the animal hepadnaviruses is quite similar. The WHV and GSHV genomes are approximately 3300 base pairs in size, and that of DHBV is approximately 3000 base pairs in size. The genomic organization of the various hepadnaviruses is similar, and there is extensive nucleotide homology among them. The mammalian hepadnaviruses differ from the avian hepadnaviruses in that the avian hepadnaviruses do not contain the X region.

HBV DNA can be found either free or integrated in the host chromosome of the hepatocyte. Free HBV DNA represents intermediate forms of replication for the viral genome and can be detected during acute infections and some chronic stages of HPV infection. Integrated sequences are usually found during chronic virus infection and in HCC. The replication mechanism for the hepadnaviruses, first discovered by Summers and Mason for DHBV[111] and later confirmed for HBV, is different from that of other DNA viruses. The replication cycle involves a reverse transcription step resembling that of the retroviruses in that it goes through an RNA copy of the genome as an intermediate in replication. The hepadnaviruses differ from the retroviruses, however, in that retrovirus virions contain RNA and the intermediate form of replication is integrated DNA. The virions of the hepadnaviruses contain DNA and the intermediate replication form is RNA. Also, integration of the hepadnavirus genome as a provirus is not a necessary intermediate step for viral genome replication as it is for a retrovirus. The similarity between the retroviruses and the hepadnaviruses extends to the overall genomic organization, in which all of the genes are encoded on only one strand. The order of the genes within the retroviruses (gag, pol, and env) is similar to their counterparts for the hepadnaviruses (core, polymerase, and surface antigen). Other subtle differences in the transcriptional programs used to generate the messenger RNAs for these different viruses exist. A further similarity between these viruses is that some members of each group of these viruses encode transcriptional regulatory factors. For HTLV-1, described later in this chapter, the X region encodes the transcriptional activator tax as well as the rex gene product involved in messenger RNA transport to the cytoplasm. The X genes encoded by the mammalian hepadnaviruses similarly encode a protein that has been extensively studied and shown to have a variety of activities, including the ability to function as a transcriptional activator. The function of X in the life cycle of the mammalian hepadnaviruses, however, is still not well understood. Although there have been studies claiming that the X protein has oncogenic properties, the evidence implying a direct role for the X protein in HCC is far from compelling.[110]

Primary infection with HBV results either in a subclinical infection or acute hepatitis B, depending on the age of the individual, among other factors. In adults, 95% of such infections resolve, with clearance of virus from the liver and the blood and with lasting immunity to reinfection. The remaining 5% of infections do not resolve but develop into a persistent hepatitis with a viremia that usually lasts for the life of the host and can have a variety of pathologic consequences. Many of these persistent infections have little associated hepatocellular injury. Approximately 20% to 25% of persistently infected individuals do develop hepatocellular injury, either chronic persistent hepatitis (in which case the inflammation is limited to periportal areas) or chronic active hepatitis (where there is inflammation and hepatocellular necrosis extending outside of the portal areas). Chronic active hepatitis has significant potential for progression to cirrhosis, hepatic failure, and cancer.

HBV and Hepatocellular Carcinoma

An etiologic role of HBV in HCC is now established. There is a striking correlation between the worldwide geographic incidence of HCC and the prevalence of HBsAg chronic carriers, and important evidence for the role of HBV in HCC was provided by the prospective epidemiologic studies of Palmer Beasley in Taiwan.[108] The classic studies from Beasley demonstrated that chronic HBV carriers in Taiwan had more than 100 times the risk of noncarriers for the development of HCC. In areas such as Taiwan that are endemic for HBV, infection with the virus occurs in early childhood, and there is an interval of approximately 30 years before the development of HCC.

Despite the strong epidemiologic evidence establishing HBV as the major cause of HCC worldwide, a mechanistic role for HBV in HCC is not fully understood. Usually in HBV-positive liver cancers, viral DNA sequences can be found integrated into the host cellular DNA. Different tumors display different patterns of integration, indicating that the insertion of the viral DNA into the host chromosome is not site specific. In a given tumor, however, all cells have the same pattern of HBV DNA integration, indicating that the integration event preceded the clonal expansion of the tumor. This clonal pattern of HBV DNA integration supports the etiologic role of HBV in HCC.

The HBV integrated genomes are often highly rearranged within tumors, displaying a variety of deletions, inversions, and point mutations. Although occasional integrated genomes do retain one or more viral genes intact, there does not appear to be a consistent pattern in which one gene is regularly preserved intact. This indicates that the continued expression of a specific viral gene is not required for the maintenance of the malignant phenotype in an HBV-positive liver cancer.

There are two hypotheses to explain how HBV causes cancer, one involving a direct role of the virus in carcinogenesis and the other indirect, as a consequence of persistent liver injury caused by the immune response to infected hepatocytes by cytotoxic T cells. The direct models imply an oncogenic role for an HBV either through the integration of the viral genome or from the oncogenic activity of a viral gene product. The indirect models do not require a direct genetic contribution by the virus or its gene products to the transforming event.

Mechanisms by which HBV DNA integration could directly contribute to tumorigenesis could be either (1) proto-oncogene activation as a result of the insertion of the viral DNA or (2) the inactivation of tumor suppressor alleles by such integration. Indeed, there is compelling evidence that insertional activation in WHV-induced hepatomas is important in hepatocellular carcinogenesis in the woodchuck model. Approximately 20% of the tumors show WHV DNA inserted into the N-myc locus.[112] This gene, normally silent in adult liver, is strongly upregulated by this insertion, and this activation can be seen early in the oncogenic sequence—even in premalignant lesions. Whereas insertional activation of N-myc clearly plays a major role in WHV oncogenesis, a similar claim cannot be made for HBV. Human hepatomas do not harbor N-myc rearrangements. An extensive search for comparable events in HBV-associated human HCC have, however, turned up only rare examples of integration in loci that might contribute to the tumorigenesis described (i.e., insertions near loci for retinoid receptors, erb-A, or cyclin As).[110] In conclusion, although insertional mutagenesis or specific oncogene activation may be important in individual cases of HCC, there is little evidence that it is of general mechanistic importance for HCC in humans.

There is also no strong evidence that HBV encodes a transforming protein. The best candidate may be the viral X protein, a small regulatory protein that is encoded by the oncogenic mammalian hepadnaviruses but not by the non-oncogenic avian hepadnaviruses. Indeed, transgenic mice with high levels of hepatic expression of X develop HCC with increased frequency.[113] Tumors in these mice do not begin until midlife, suggesting that additional genetic changes are necessary for cancer development. The X protein has no homology to known oncogenes or cellular genes involved in signaling or growth control. X has been extensively studied from a functional standpoint, but its precise role in the hepadnavirus life cycle remains unclear. The X protein can stimulate cytoplasmic signal transduction pathways (e.g., the ras-raf MAP kinase pathway), can also function as a nuclear transcriptional activator, and can interfere with cellular DNA repair by binding DNA repair proteins.[110] The relationship of all of these activities to the putative oncogenic function of X is unproven. It may be that the role of the HBV X gene product in tumorigenesis in the transgenic mouse lines that have been derived is an indirect one, possibly due to liver injury and triggering hepatocellular regeneration from the overexpression of the X protein. It should be noted further that the X protein is not always expressed in HBV-positive HCCs.

Despite the absence of strong data to support a direct oncogenic role for HBV in HCC pathogenesis, there is mounting support for a more indirect model, in which neither the HBV genome nor any of its products make a direct genetic contribution to the transformation of the infected cell. Instead, HBV-induced cellular injury, a consequence of the immune or inflammatory responses to HBV infection, results in liver cell regeneration that, over time, can lead to cancer. Cellular proliferation in liver regeneration increases the chances for errors in DNA replication, leading to mutations that can contribute to the loss of normal cellular growth control. Those cells with an appropriate set of genetic mutations can then undergo clonal expansion and ultimately progress to HCC. In this indirect model, HBV promotes oncogenesis chiefly by provoking cellular proliferation in response to immune-mediated injury. Thus no direct genetic contribution is made by viral sequences acting in cis or viral gene products acting in trans. Significant experimental support for the indirect model for HBV-induced carcinogenesis has come from important experiments in HBV transgenic mice.[114-116]

Even though the tumorigenic mechanisms of HBV-induced carcinogenesis remain unclear, the overwhelming epidemiologic data clearly establish HBV as the principal cause of most cases of HCC worldwide. An effective vaccine to prevent HBV infection is predicted to prevent the majority of HCC. Vaccines expressing the surface antigens produced by recombinant techniques in yeast or in Chinese hamster ovary cells have been developed commercially, are highly immunogenic, and can protect against HBV infection.[117] The efficacy of protection against HBV infection has been established in large clinical studies of high-risk individuals. The reduction in the levels of HCC following a reduction in the HBV carrier rates among the vaccinated populations has provided confirmation of the role of HBV in HCC.[118] A universal HBV vaccination program has been

recommended by the World Health Organization, with the intended goal of eradicating HBV.

Hepatitis C Virus

Hepatitis C virus (HCV) is a human flavivirus, a positive-strand RNA virus that is an important cause of morbidity and mortality worldwide. HCV carries a very high rate of chronicity after infection, with over 70% of those infected going on to develop chronic liver disease. HCV is believed to be the leading infectious cause of chronic liver disease in the Western world. It is also etiologically responsible for many cases of HCC worldwide. Between 30% and 70% of HBV-negative HCC patients are seropositive for HCV. In the United States, it appears that as many as 40% of the cases of HCC may be associated with HCV. HCV positivity conveys about an 11.5-fold increased risk for the development of liver cancer.[119]

HCV was first cloned in 1989 from the infectious sera of individuals with posttransfusion hepatitis.[120,121] Much of our knowledge of this virus derives from molecular genetic and biochemical studies because, until recently, there were no suitable tissue culture systems or animal models for the study of this virus. Nonetheless, there have been major advances in our understanding of the molecular biology of this important human pathogen.[122] HCV is a single positive-stranded RNA virus with a 9.4-kb RNA genome that contains a single ORF encoding a polyprotein of 3011 amino acids. This large polyprotein is then posttranslationally cleaved to produce several mature structural and nonstructural proteins. The HCV virus is inherently unstable, giving rise to multiple types and subtypes. This genome instability is due to the dependence of the virus on the virally encoded RNA-dependent RNA polymerase to perform the RNA-to-RNA copying of the genome. There is no DNA intermediate in the replication of the genome, excluding the possibility of viral genome integration as a mechanism for HCV-associated carcinogenesis. Furthermore, the polymerase lacks proofreading capability, and there is a substantial level of base misincorporation, accounting for the marked heterogeneity in viral isolates even from a single infected individual. There is a high degree of variability in the viral envelope glycoproteins, which has led to the hypothesis that changes in these genes alter the antigenicity of the virus over time, permitting the virus to escape immune recognition by the host. This variability allowing the virus to escape the immune system is important to the pathogenesis of the virus in establishing a persistent infection. A characteristic feature of an HCV infection is repeated episodes of hepatic damage, resulting from the reemergence of a newly mutated genotype. This genomic heterogeneity, due to the ability of the virus to rapidly mutate, has proved problematic in attempts to develop an effective vaccine to HCV.

It is unclear whether HCV contributes directly to hepatocarcinogenesis. As noted earlier, HCC does not replicate through a DNA intermediate and therefore cannot integrate into host chromosomes, causing insertional mutagenesis. The virus encodes several nonstructural (NS) proteins that are involved in viral genome replication and in altering the cell environment to allow a persistent infection. For instance, one of these proteins, NS5A, can affect interferon signaling and cellular apoptosis through interactions with specific cellular proteins. Interactions of some of the HCV NS proteins with cellular proteins involved in cellular tumor suppression pathways have been described, and there have been a few reports suggesting some oncogenic properties for the viral NS proteins in transfection experiments.[122] To date, however, there is no compelling body of evidence suggesting that HCV encodes a protein that directly contributes to HCC development. Instead, as with HBV, the bulk of the data suggests that the role of HCV in hepatocarcinogenesis may be indirect, through persistent infection, chronic inflammation, and cirrhosis.

Human Retroviruses and Cancer

Human T-Cell Leukemia Viruses

The first substantiated reports of a human retrovirus were published in 1980 and 1981 from Robert Gallo and his colleagues at the NCI[123,124] and soon after from Yoshida and his colleagues in Japan.[125] These isolates were from human T-cell leukemia cell lines. The human T-cell leukemia virus type 1 (HTLV-1) is recognized as the etiologic agent of adult T-cell leukemia (ATL). A causal relationship between HTLV-1 and ATL was initially suggested by epidemiologic studies showing geographic clustering of ATL, a pattern that is consistent with an infectious agent. A second human retrovirus, referred to as HTLV-2, was initially isolated in 1982 from a cell line established from a patient with an unusual form of hairy cell leukemia.[126] However, studies have not established an association of HTLV-2 with any human malignancy.

ATL was first described by Takatsuki and his colleagues in 1977,[127] before the virus was discovered. It is a malignancy of mature CD4-positive lymphocytes and is endemic in parts of Japan, as well as in the Caribbean and in parts of Africa. Clinically the tumor resembles mycosis fungoides and Sezary syndrome but is more aggressive than these other two syndromes, with a median survival from the time of diagnosis of only 3 to 4 months. In addition to skin involvement, it affects visceral organs and there is often an associated hypercalcemia.

Serologic assays specific for HTLV-1 viral antigens revealed that virus infection is more widespread in

the endemic areas than is ATL.[128] It is estimated that an HTLV-1–infected individual has about a 3% lifetime risk of developing ATL. HTLV-1 infection is most marked in the southernmost islands of Japan and the Caribbean. Parts of Africa appear to have the next largest reservoirs of infection. The prevalence in the United States and in Europe is low in the general population, although it is quite high among intravenous drug abusers. A preleukemic disease in the form of a chronic lymphocytosis often precedes the development of acute leukemia or lymphoma. ATL usually occurs in early adulthood, and this is believed to be approximately 20 to 30 years after the initial infection in the subset of individuals who develop it.

HTLV-1 infection has been associated with a second clinical entity: HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic degenerative neurologic syndrome that primarily affects the spinal cord. Specific risk factors that may be important in determining the development of ATL or TSP in the HTLV-1–infected individual are currently not known. Transmission, when it occurs in childhood, is usually from the mother through breast milk and can result in ATL in a small percentage of patients as adults several decades later. The factors that contribute to disease progression in the few percent of HTLV-1–infected individuals who will develop ATL are not known. HAM/TSP, on the other hand, usually occurs in individuals through parenteral transmission by blood transfusion or intravenous drug use or through sexual transmission. It is generally believed that HAM/TSP is primarily the result of an autoimmune process against the central nervous system somehow initiated by the viral infection.

Epidemiologic studies have shown that about 2% to 5% of individuals seropositive for HTLV-1 will develop ATL. The virus is transmitted from mother to infants through mother's milk and in adults is transmitted through sexual contact and through contaminated blood. The latency period between the time of infection and the development of ATL can vary from a few years to as long as 40 years. There is some evidence to suggest that the virus's role in leukemogenesis may be direct, in that the virus alone appears to be sufficient to initiate a series of events that may lead to leukemia independent of subsequent environmental factors.

Molecular studies suggest a possible direct role of HTLV-1 as an etiologic agent in ATL. In the life cycle of a retrovirus, the provirus (i.e., the double-stranded DNA copy of the viral RNA genome) becomes integrated into the cellular genome at random positions as part of the life cycle of the virus. In the leukemic cells of an ATL patient, however, the viral sequences are found integrated in the same place in each cell, although the site of integration varies from leukemia to leukemia. This indicates that ATL is clonal and all of the leukemic cells must necessarily derive from a single cell, and that the viral infection must have preceded the expansion of the tumor.

HTLV-1 is also a transforming virus capable of immortalizing normal human umbilical cord blood lymphocytes (T-cells) in vitro. The mechanism by which HTLV-1 induces leukemogenesis is different from that of the other chronic leukemia retroviruses studied in animals, such as the avian leukosis virus or the murine leukemia virus.

The fact that the HTLV-1 provirus integration site varies from leukemia to leukemia is consistent with the HTLV-1 genome encoding a factor that is critical in the early stages of leukemogenesis. HTLV-1 and its relative HTLV-2 belong to a distinct group of retroviruses that has been referred to as trans-regulating retroviruses and includes the bovine leukemia virus, the biology of which is actually quite similar to that of HTLV-1 and HTLV-2. This group of retroviruses differs from the chronic leukemia viruses and the acute leukemia viruses as depicted in Figure 6-5 by the fact that they contain additional genes at the 3′ end of the genome. This region is called the X region and encodes trans-regulatory factors involved in transcriptional activation, translational control, and mRNA transport from the nucleus. Two unique regulatory genes, tax and rex, encoded by this region have been particularly well studied.[129] The tax gene serves as a master key for activating transcription from the viral long terminal repeat (LTR), and the rex gene is involved in the transport of specific viral messenger RNA species from the nucleus to the cytoplasm.

There is good evidence supporting a direct role for tax as a transforming gene in the causation of ATL. Tax can immortalize human CD4-positive T cells in an IL-2–independent manner, transform rodent fibroblasts in tissue culture, and induce tumors in transgenic mice.[129] Multiple transforming activities of Tax have been described that have been linked to its ability to either activate specific cellular transcription factors or affect the cell cycle through interactions with cell-cycle-inhibitors and inhibiting apoptosis and cellular DNA repair.

Tax transactivates the viral LTR promoter through its interaction with CREB/ATF-1, CBP/p300, and the Tax-responsive 21-bp repeat element (TRE).[129] In addition, the tax gene product has been shown to activate transcription of specific cellular genes, including lymphokines, the IL-2 gene, and the IL-2 receptor gene through the NFκB pathway.[130] It is felt that HTLV-1 may initiate the leukemogenic process through activation of specific cellular genes by tax. One mechanism by which HTLV-1 could induce cellular proliferation and immortalization could involve an autocrine loop through the tax-mediated stimulation of both IL-2 and its receptor. Tax-mediated activation of cellular genes may also involve paracrine mechanisms. Tax has also been shown to activate the expression of a group of nuclear oncogenes,

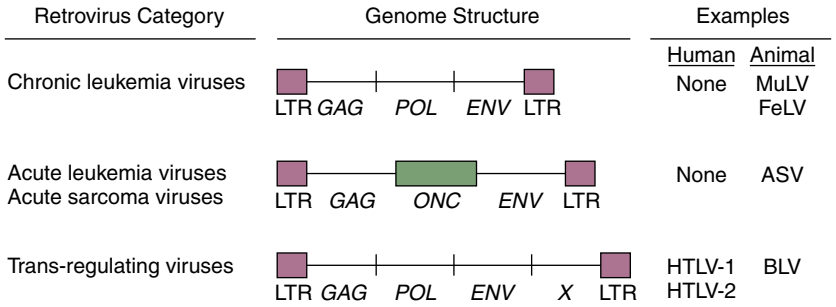


Figure 6-5 The genomic organization of different types of retroviruses The prototype retrovirus represented in the figure by the chronic leukemia viruses. It contains regulatory sequences at each end derived from the long terminal repeat (LTR) elements of the virus as well as the coding sequences for the viral proteins gag, pol, and env. The acute transforming retroviruses are defective viruses. Acquired onc sequences from the cellular genome replace critical viral gene segments. These defective viruses can therefore only replicate in the presence of a replication-competent helper virus. The trans-regulatory retroviruses contain sequences, 3′ to the env gene, that encode regulatory factors. This region has been referred to as the X region and encodes the tax and rex genes.

including c-fos, c-egr, and c-jun.[129] The mechanism by which Tax activates these various cellular promoters is through interactions with cellular transcription factors. The factors identified include CREB and the CRE modulator protein (CREM), the NFκB family of proteins, and the serum response factor (SRF).

Tax also binds and inactivates the inhibitory proteins of NFκB called IκB.[131,132] There is a complex of IκB proteins, most notably IκBα, that bind and retain NFκB in the cytoplasm until there is a signal for activation, when IκBα is targeted for proteolysis, releasing NFκB to translocate into the nucleus to activate transcription of its downstream effectors. Through binding IκB, Tax destabilizes the IκB/NFκB complex and activates NFκB. Thus the Tax mechanism of activation of genes under the control of NFκB appears to be two pronged, first, through the suppression of its cytoplasmic tether, IκB, and second through binding NFκB directly and bridging it with the basic transcriptional machinery.

In addition to its transcriptional activation functions, Tax also affects many aspects of the cell cycle. Tax can complex with p16INK4A, a cell cycle inhibitor, which binds and inhibits the activity of cell cycle–dependent kinase 4 (cdk4).[133] Cdk4 works with cyclin D to phosphorylate and inactivate the retinoblastoma protein (pRB). The consequence of Tax inactivation of p16INK4A is therefore the activation of cyclin D/cdk4 and the inactivation of pRB, which in turn leads to cell cycle activation, driving the proliferation of cells from G_1 to S. The pathway regulating pRB is commonly targeted by the DNA tumor virus oncoproteins, as discussed earlier in this chapter. In addition, Tax is capable of inactivating p53 functions,[134] inducing p21*CIP* expression,[135] and inhibiting apoptosis and DNA repair.[136] In addition, Tax can dramatically perturb mitotic regulation, causing micronuclei formation, cytokinesis failure, and chromosome instability.[137] HTLV-1 also encodes a protein on its minus strand called the HTLV-1 basic leucine zipper factor (HBZ) that may also be important to the leukemogenic activity of the virus. HBZ is expressed in ALT cells and promotes proliferation of T cells.[138]

The activities of Tax and HBZ are likely important for the direct role of HTLV-1 in the initiation and progression of leukemogenesis.[129]

HIV, AIDS, and Cancer

The human immunodeficiency viruses (HIV-1 and HIV-2) are members of a distinct subclass of retroviruses called lentiviruses.[139] Similar to HTLV-1, the HIVs also infect CD4-positive T lymphocytes. Beyond sharing a common cellular host for replication, however, the viruses are not closely related and do not share any serologic cross reactivity. HIV-1 and HIV-2 are associated with AIDS. These viruses themselves do not appear to play a major direct etiologic role in any specific human tumors. Patients with AIDS, however, do have a high incidence of specific tumors including KS and other cancers that are often caused by specific viruses.[140] Indeed, one of the earliest diagnostic features of AIDS in homosexual males can be KS, a tumor that was regarded as extremely rare before the current AIDS epidemic. The etiology of KS involves the Kaposi's sarcoma herpesvirus (KSHV or HSV-8) and is due to the uncontrolled proliferation of an activated microvascular endothelial cell, which is believed to be the cell of origin in KS (see earlier section on KSHV).

Other tumors often seen in AIDS patients include non-Hodgkin's lymphomas and papillomavirus-associated cancers, including anal squamous cell carcinomas and cervical cancer. Because of the immunodeficiency in AIDS patients, viral infections are common, and some of the tumors seen in these patients likely have a viral etiology. For instance, a high percentage of AIDS patients develop lymphomas, including central nervous system lymphomas. Some, but not all, of these lymphomas may be accounted for in part by the emergence of populations of B lymphocytes transformed by EBV. It is also possible that HTLV-1 may account for some

lymphomas in patients with AIDS. AIDS patients are often infected by papillomaviruses and HBV. The genital warts, anal and anal squamous cell carcinomas, and cervical cancers seen in these patients are due to the specific HPV types that are oncogenic in immunocompetent individuals.

Human Polyomaviruses and Cancer

SV40

There have been periodic reports dating back to the 1970s claiming the presence of SV40 DNA of SV40 in a variety of different human cancers, including osteosarcomas, mesotheliomas, pancreatic tumors, and brain tumors. A potential role of SV40 in some human cancers has been a very controversial area that has received scrutiny from investigators in the field and from the National Cancer Institute. I will not summarize all of these studies, but instead refer the reader to a recent review of this controversial subject.[141,142]

SV40 is a nonhuman primate virus that naturally infects Asian macaques. The major source of human exposure to SV40 was through contaminated poliovirus vaccines that were given between 1955 and 1963. SV40 is a highly oncogenic virus in rodent cells and has served as an extremely valuable model for determining the various mechanisms by which DNA tumor viruses transform cells and contribute to tumor formation. However, there is no epidemiologic evidence indicating a higher risk of cancers among the populations of individuals who received the SV40-contaminated vaccine.

There is also no compelling data that the virus is circulating among human communities. SV40 is closely related to the human polyomaviruses BK and JC, and much of the seroreactivity to SV40 seen in humans can be accounted for by cross reactivity with BK and/or JC virus. In addition, much of the data claiming an association of SV40 DNA with human tumors have been gathered by the use of PCR assays, which are error prone, and have been difficult to confirm. Furthermore, the PCR primers used in many of these studies would detect sequences that are present in many laboratory plasmid vectors, raising the possibility of laboratory contamination. Indeed, studies suggest that flawed PCR detection methodologies and laboratory plasmids might have contributed significantly to the positive claims for SV40 tumor associations.[143,144]

BKV and JCV

There have been periodic claims that the human polyomaviruses BK and JC are also associated with specific human cancers. Infections with both of these viruses are widespread in humans as measured by seroreactivity. They encode tumor (T) antigens similar in function to SV40 large T antigen and can functionally inactivate the p53 and pRB pathways. For JC virus, which is the cause of progressive multifocal leukoencephalopathy (PML), there have been reports of DNA and T antigen in brain tumors of patients with or without PML. This is a provocative association that will need confirmation and validation. A number of studies have found an association of BK virus with a variety of different types of cancers as well as precancerous lesions of the prostate. The presence and potential role of these viruses in the cancers with which they have been found will also need to be further explored.

Merkel Cell Polyomavirus

In 2008, Yuan Chang and Patrick Moore identified a new human polyomavirus by deep sequencing techniques that they called the Merkel cell polyomavirus (MCV) in human Merkel cell carcinomas (MCC).[145] MCC is a relatively rare cancer in humans that was first described in 1972 by Cyril Toker.[146] MCC is an aggressive tumor seen in immunosuppressed individuals and the elderly. MCV DNA is clonally integrated into the positive cancers, suggesting an etiologic role in the cancers. Furthermore, the expression of the virally encoded tumor antigens is required to maintain the cancer cells in tissue culture, strongly implicating the virus as the cause of these cancers.

Like the other human polyomaviruses, MCV is quite common, and initial infection occurs in childhood.[147] MCV can be found on the skin of healthy individuals[148] and appears ubiquitous in the human population. MCV DNA and its expression can be found in over 80% of MCCs, supporting an active role of the viruses in these cancers. Only a portion of the early region encoding the first half of the large T antigen as well as the small t antigen is expressed in the MCV-positive cancers, as recently reviewed.[149] Molecular biology research on MCV is a very active area of research to identify the mechanisms by which MCV transforms cells and contributes to cancer. There are also studies ongoing to determine whether MCV is associated with human cancers in addition to MCC.

Bacteria and Cancer

Helicobacter pylori and Gastric Cancer

Helicobacter pylori entered the scientific lexicon during the mid-1980s with the work of Robin Warren and Barry Marshal, who first cultured the bacterium and determined that it was the causative agent of most gastric and duodenal ulcers.[150,151] For their work, they shared the 2005 Nobel Prize in medicine, as well as upending the notion that gastric ulcers were mainly caused by stress and diet.

H. pylori (first known as *Campylobacter pyloridis*) is a gram-negative, flagellated spiral or curved bacillus that colonizes the stomach via attachment to gastric epithelial

cells. The complete genome was sequenced in 1997[152] and predicted to encode for approximately 1500 ORFs, many involved in adaptation for growth in the inhospitable acidic environment of the stomach. Infection is found in over 80% of the worldwide population, although a much smaller population develops gastric ulcers due to infection.

Two human cancers have been correlated with *H. pylori* infection: gastric cancer and MALT (*m*ucosa-*a*ssociated *l*ymphoid *t*issue) lymphoma of the stomach. The correlation was strong enough that *H. pylori* was categorized as a carcinogen by the International Agency for Research on Cancer (IARC). Gastric cancers are the fourth most common cancer worldwide. Since the isolation of the organism and the sequencing of its genome, a number of potential transformation mechanisms have been proposed, involving both epithelia and immune cell populations. There are a number of possible mechanisms related to *H. pylori*–induced transformation. One observation is that *H. pylori* produces excess free radicals, leading to host cell DNA damage and the accumulation of host cellular mutations.

A potentially oncogenic factor produced by *H. pylori* is the CagA protein. CagA is injected in gastric epithelial cells via type-IV secretion and has been shown to alter a number of signal transduction pathways.[153] One target of CagA is SHP-2, a tyrosine phosphatase implicated in some human cancers. CagA induces SHP-2 activation, leading to disruptions in cell adhesion and cell junctions and an increase in cell motility. Another factor produced by the bacterium is VacA, a secreted vacuolating cytotoxin protein that inhibits the ability of T lymphocytes to neutralize infection and allows the bacterium to evade the immune system and set up a chronic infection. Another proposed mechanism of *H. pylori*–induced transformation has been called a perigenetic pathway which refers to the effect that chronic inflammation has on host epithelial cells (reviewed in Ref. 154). Infection can induce TNF-α and IL-6, which can alter host cell adhesion and lead to migration of mutated cells.

Parasites and Cancer

Parasites were perhaps the first infectious agents to be potentially linked with human cancer. In 1900, Askanazy reported a link between *Opisthorchis felineus* infection and liver cancer, and Goebel published a report incriminating *Bilharzia* infections (schistosomiasis) with human bladder cancer.[155] Indeed, the Nobel Prize in medicine was awarded to Johannes Fibiger in 1926 for studies linking a nematode with tumors in rats; however, those studies could not be reproduced. Today there are two well-established associations of parasites with human cancer: schistosomiasis with bladder cancer and liver flukes with cholangiocarcinoma. The major burden for parasite-associated cancers is in developing countries.

Schistosomiasis and Bladder Cancer

Schistosomiasis, also known as bilharzia, is a parasitic disease caused by trematodes from the genus *Schistosoma*. *S. haematobium* is responsible for urinary schistosomiasis and can cause chronic infections that can lead to kidney damage and to bladder cancer. *S. haematobium* infections are a significant public health problem in much of Africa and the Middle East, second only to malaria among parasitic diseases. Bladder cancers associated with *S. haematobium* are squamous cell cancers and are histologically different from the transitional-cell carcinomas that are more commonly seen in the United States and Europe. The mechanism by which *S. haematobium* causes bladder cancer is unknown but most likely is a consequence of a persistent, chronic infection.

Liver Flukes and Cholangiocarcinoma

Opisthorchis viverrini and *Clonorchis sinensis* are liver flukes (a type of flatworm) that are associated with an increased risk of cholangiocarcinomas. Infections with these liver flukes come from eating raw or undercooked fish. They occur almost exclusively in East Asia and are rare in other parts of the world. Cholangiocarcinoma is more common in areas of endemic liver fluke infection (Hong Kong, Thailand). *O. viverrini* is endemic in northeast Thailand and is estimated to infect approximately 9 million people. *C. sinensis* infects approximately 7 million people in China and other parts of the Far East. Liver flukes usually enter the human gastrointestinal tract after ingestion of raw fish, and the parasites then travel via the duodenum into the host's intrahepatic or extrahepatic biliary ducts. Liver flukes cause bile stasis, inflammation, periductal fibrosis, and hyperplasia, with the subsequent development of cholangiocarcinoma.

Perspectives

Infectious agents play a major role in human cancer, either as direct or indirect carcinogens. In this chapter I have reviewed the association of a number of agents that have been generally accepted as playing a major role in human cancer. I have also discussed the controversy surrounding SV40 as a potential oncogenic agent. There are other agents that have been implicated in the literature but for which the data are not yet compelling. Individual infectious agents

contribute to carcinogenesis in different ways. Some, like HPV and cervical cancer or HTLV-1 and ATL and perhaps MCV and MCC, do so in a direct manner through oncogenic proteins encoded by the virus. Others, like the liver flukes and cholangiocarcinomas and *S. haematobium* and bladder cancer, likely do so through indirect mechanisms involving persistent infection and inflammation. The criteria that are generally used to determine whether or not an agent is carcinogenic must therefore necessarily involve a combination of epidemiology and molecular biology. Several questions arise. Are there additional unknown infectious agents associated with human cancer? If so, what cancers, and how can they be discovered? Certainly cancers in immunologically compromised individuals are good candidates for an infectious etiology. Also, advances in array technologies and bioinformatics searching tools should provide important platforms to examine such cancers. Another question is whether some very ubiquitous infectious agents might contribute to the initiation of some cancers, but do so in a hit-and-run fashion such that a molecular fingerprint is not left behind. How will the role of such agents in human cancers be discovered?

Key References

2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005;55:74-108.
12. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine*. 2012;30(suppl 5):F55-F70.
13. Howley PM, Schiller JT, Lowy DR. Papillomaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. 6th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1662-1703.
17. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol*. 2012;13:607-615.
18. Freeman H, Wingrove B. *Excess Cervical Cancer Mortality: A Marker for Low Access to Health Care in Poor Communities*. Rockville, Md: National Cancer Institute, Center to Reduce Cancer Health Disparities; 2005 NIH Pub. No. 05–5282.
20. Durst M, Gissmann L, Idenburg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A*. 1983;80:3812-3815.
22. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 2003;348:518-527.
23. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer*. 2010;10:550-560.
24. White EA, Howley PM. Proteomic approaches to the study of papillomavirus-host interactions. *Virology*. 2013;435:57-69.
26. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol*. 2004;78:11451-11460.
27. Korzeniewski N, Spardy N, Duensing A, Duensing S. Genomic instability and cancer: lessons learned from human papillomaviruses. *Cancer Lett*. 2011;305:113-122.
34. Pihan GA, Wallace J, Zhou Y, Doxsey SJ. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res*. 2003;63:1398-1404.
42. Huang LW, Chao SL, Chen TJ. Reduced Fhit expression in cervical carcinoma: correlation with tumor progression and poor prognosis. *Gynecol Oncol*. 2003;90:331-337.
43. Yang YX, Yang AH, Yang ZJ, Wang ZR, Xia XH. Involvement of tumor suppressor in lung cancer 1 gene expression in cervical carcinogenesis. *Int J Gynecol Cancer*. 2006;16:1868-1872.
44. Backes DM, Kurman RJ, Pimenta JM, Smith JS. Systematic review of human papillomavirus prevalence in invasive penile cancer. *Cancer Causes Control*. 2009;20:449-457.
45. Smith JS, Backes DM, Hoots BE, Kurman RJ, Pimenta JM. Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors. *Obstet Gynecol*. 2009;113:917-924.
49. Park IU, Palefsky JM. Evaluation and management of anal intraepithelial neoplasia in HIV-negative and HIV-positive men who have sex with men. *Curr Infect Dis Rep*. 2010;12:126-133.
50. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011;29:4294-4301.
51. Jordan RC, Lingen MW, Perez-Ordonez B, et al. Validation of methods for oropharyngeal cancer HPV status determination in U.S. cooperative group trials. *Am J Surg Pathol*. 2012;36:945-954.
52. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363:24-35.
53. Ramqvist T, Dalianis T. An epidemic of oropharyngeal squamous cell carcinoma (OSCC) due to human papillomavirus (HPV) infection and aspects of treatment and prevention. *Anticancer Res*. 2011;31:1515-1519.
60. Orth G. Human papillomaviruses associated with epidermodysplasia verruciformis in non-melanoma skin cancers: guilty or innocent? *J Invest Dermatol*. 2005;125:xii-xiii.
61. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*. 2007;370:59-67.
62. Feltkamp MC, de Koning MN, Bavinck JN, Ter Schegget J. Betapapillomaviruses: innocent bystanders or causes of skin cancer. *J Clin Virol*. 2008;43:353-360.
64. Bouwes Bavinck JN, Neale RE, Abeni D, et al. Multicenter study of the association between betapapillomavirus infection and cutaneous squamous cell carcinoma. *Cancer Res*. 2010;70:9777-9786.
65. Proby CM, Harwood CA, Neale RE, et al. A case-control study of betapapillomavirus infection and cutaneous squamous cell carcinoma in organ transplant recipients. *Am J Transplant*. 2011;11:1498-1508.
67. Weissenborn SJ, Nindl I, Purdie K, et al. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J Invest Dermatol*. 2005;125:93-97.
68. Arron ST, Ruby JG, Dybbro E, Ganem D, Derisi JL. Transcriptome sequencing demonstrates that human papillomavirus is not active in cutaneous squamous cell carcinoma. *J Invest Dermatol*. 2011;131:1745-1753.
69. Lowy DR, Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest*. 2006;116:1167-1173.
70. Schiller JT, Castellsague X, Villa L, Hildesheim A. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. *Vaccine*. 2008;26S:K53-K61.
71. Brown DR, Kjaer SK, Sigurdsson K, et al. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16-26 years. *J Infect Dis*. 2009;199:926-935.
72. Herrero R, Wacholder S, Rodriguez AC, et al. Prevention of persistent human papillomavirus infection by an HPV16/18 vaccine: a community-based randomized clinical trial in Guanacaste, Costa Rica. *Cancer Discov*. 2011;1:408-419.
73. Wheeler CM, Castellsague X, Garland SM, et al. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. *Lancet Oncol*. 2012;13:100-110.

74. Chaturvedi AK. Beyond cervical cancer: burden of other HPV-related cancers among men and women. *J Adolesc Health*. 2010;46:S20-S26.
75. Peres J. For cancers caused by HPV, two vaccines were just the beginning. *J Natl Cancer Inst*. 2011;103:360-362.
76. Pastrana DV, Gambhira R, Buck CB, et al. Cross-neutralization of cutaneous and mucosal papillomavirus types with anti-sera to the amino terminus of L2. *Virology*. 2005;337:365-372.
77. Roden RB, Ling M, Wu TC. Vaccination to prevent and treat cervical cancer. *Hum Pathol*. 2004;35:971-982.
82. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet*. 1964;1:702-703.
84. Longnecker R, Kieff E, Cohen JI. Epstein-Barr virus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1898-1959.
104. Cesarman E, Moore PS, Rao PH, Inghirami G, Knowles DM, Chang Y. In vitro establishment and characterization of two AIDS-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood*. 1995;86:2708-2714.
107. Damania B, Cesarman E. Kaposi's sarcoma-associated herpesvirus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:2080-2128.
110. Seeger C, Zoulim F, Mason WS. Hepadnaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:2185-2221.
117. Beasley RP. Hepatitis B virus—the major etiology of the hepatocellular carcinoma. *Cancer*. 1988;61:1942-1956.
118. Chang MH, You SL, Chen CJ, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. *J Natl Cancer Inst*. 2009;101:1348-1355.
122. Ray SC, Bailey JR, Thomas DL. Hepatitis C virus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:795-824.
124. Poiesz BJ, Ruscetti FW, Reitz MS, Kalyanaraman VS, Gallo RC. Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukemia. *Nature*. 1981;294:268-271.
129. Fujii M, Matsuoka M. Human T-cell leukemia virus types 1 and 2. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1474-1501.
134. Pise-Masison CA, Brady JN. Setting the stage for transformation: HTLV-1 Tax inhibition of p53 function. *Front Biosci*. 2005;10:919-930.
135. Kawata S, Ariumi Y, Shimotohno K. p21(Waf1/Cip1/Sdi1) prevents apoptosis as well as stimulates growth in cells transformed or immortalized by human T-cell leukemia virus type 1-encoded tax. *J Virol*. 2003;77:7291-7299.
137. Liu B, Liang MH, Kuo YL, et al. Human T-lymphotropic virus type 1 oncoprotein tax promotes unscheduled degradation of Pds1p/securin and Clb2p/cyclin B1 and causes chromosomal instability. *Mol Cell Biol*. 2003;23:5269-5281.
138. Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci U S A*. 2006;103:720-725.
141. Poulin DL, DeCaprio JA. Is there a role for SV40 in human cancer? *J Clin Oncol*. 2006;24:4356-4365.
142. Shah KV. SV40 and human cancer: a review of recent data. *Int J Cancer*. 2007;120:215-223.
143. Lopez-Rios F, Illei PB, Rusch V, Ladanyi M. Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids. *Lancet*. 2004;364:1157-1166.
144. Manfredi JJ, Dong J, Liu WJ, et al. Evidence against a role for SV40 in human mesothelioma. *Cancer Res*. 2005;65:2602-2609.
145. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science*. 2008;319:1096-1100.
147. Tolstov YL, Pastrana DV, Feng H, et al. Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. *Int J Cancer*. 2009;125:1250-1256.
148. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe*. 2010;7:509-515.
149. DeCaprio JA, Imperiale MJ, Major EO. Polyomaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1633-1661.
151. Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Med J Aust*. 1985;142:436-439.
153. Hatakeyama M. *Helicobacter pylori* CagA—a bacterial intruder conspiring gastric carcinogenesis. *Int J Cancer*. 2006;119:1217-1223.
154. Tsuji S, Kawai N, Tsujii M, Kawano S, Hori M. Review article: inflammation-related promotion of gastrointestinal carcinogenesis—a perigenetic pathway. *Aliment Pharmacol Ther*. 2003;18(suppl 1):82-89.
155. zur Hausen H. *Infections Causing Human Cancer*. Weinheim, Germany: Wiley-VCH; 2006.

This page intentionally left blank

References

1. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer*. 1999;83:18-29.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005;55:74-108.
3. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. *J Intern Med*. 2000;248:171-183.
4. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res*. 1994;305:253-264.
5. Ellermann V, Bang O. Experimentelle Leukamie bei Huhnern. *Zentralbl Bakteriol*. 1908;46:595-609.
6. Rous P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J Exp Med*. 1911;13:397-411.
7. Shope RE, Hurst EW. Infectious papillomatosis of rabbits; with a note on the histopathology. *J Exp Med*. 1933;58:607-624.
8. Rous P, Beard JW. Carcinomatous changes in virus-induced papillomas of rabbits. *Proc Soc Exp Biol Med*. 1935;32:578-580.
9. Gross L. Pathogenic properties, and "vertical" transmission of the mouse leukemia agent. *Proc Soc Exp Biol Med*. 1951;62:523-548.
10. Gross L. A filtrable agent, recovered from Akr leukemia extracts, causing salivary gland carcinomas in C3H mice. *Proc Soc Exp Biol Med*. 1953;83:414-421.
11. Stewart SE. Leukemia in mice produced by a filterable agent present in AKR leukemic tissues with notes on a sarcoma produced by the same agent. *Anat Rev*. 1953;117:532.
12. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine*. 2012;30(suppl 5):F55-F70.
13. Howley PM, Schiller JT, Lowy DR. Papillomaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. 6th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1662-1703.
14. Rous P, Kidd JG. The carcinogenic effect of a virus upon tarred skin. *Science*. 1936;83:468-469.
15. Kidd JG, Rous P. Effect of the papillomavirus (Shope) upon tar warts of rabbits. *Proc Soc Exp Biol Med*. 1937;37:518-520.
16. Jarrett WFH, McNeil PE, Grimshaw WIR, Selman IE, McIntyre WIM. High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papillomavirus. *Nature*. 1978;274:215-217.
17. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol*. 2012;13:607-615.
18. Freeman H, Wingrove B. *Excess Cervical Cancer Mortality: a Marker for Low Access to Health Care in Poor Communities*. Rockville, Md: National Cancer Institute, Center to Reduce Cancer Health Disparities; 2005 NIH Pub. No. 05–5282.
19. Meisels A, Fortin R. Condylomatous lesions of the cervix and vagina. I. Cytologic patterns. *Acta Cytol*. 1976;20:505-509.
20. Durst M, Gissmann L, Idenburg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci U S A*. 1983;80:3812-3815.
21. Boshart M, Gissman L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J*. 1984;3:1151-1157.
22. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 2003;348:518-527.
23. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer*. 2010;10:550-560.
24. White EA, Howley PM. Proteomic approaches to the study of papillomavirus-host interactions. *Virology*. 2013;435:57-69.
25. White A, Livanos EM, Tlsty TD. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. *Genes Dev*. 1994;8:666-677.
26. Munger K, Baldwin A, Edwards KM, et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol*. 2004;78:11451-11460.
27. Korzeniewski N, Spardy N, Duensing A, Duensing S. Genomic instability and cancer: lessons learned from human papillomaviruses. *Cancer Lett*. 2011;305:113-122.
28. Duensing S, Munger K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res*. 2002;62:7075-7082.
29. Münger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol*. 1989;63:4417-4421.
30. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J*. 1989;8:3905-3910.
31. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science*. 1990;248:76-79.
32. Huibregtse JM, Scheffner M, Howley PM. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J*. 1991;10:4129-4135.
33. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquination of p53. *Cell*. 1993;75:495-505.
34. Pihan GA, Wallace J, Zhou Y, Doxsey SJ. Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res*. 2003;63:1398-1404.
35. Gardiol D, Kuhne C, Glaunsinger B, Lee SS, Javier R, Banks L. Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene*. 1999;18:5487-5496.
36. Nakagawa S, Huibregtse JM. Human scribble (vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol*. 2000;20:8244-8253.
37. Klingelhutz AJ, Foster SA, McDougall JK. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature*. 1996;380:79-81.
38. Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature*. 1998;396:84-88.
39. Winkelstein WJ. Smoking and cancer of the uterine cervix. *Am J Epidemiol*. 1977;106:257-259.
40. Yokota J, Tsukada Y, Najajima T, et al. Loss of heterozygosity on the short arm of chromosome 3 in carcinoma of the uterine cervix. *Cancer Res*. 1989;49:3598-3601.
41. Ohta M, Inoue H, Cotticelli MG, et al. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell*. 1996;84:587-597.
42. Huang LW, Chao SL, Chen TJ. Reduced Fhit expression in cervical carcinoma: correlation with tumor progression and poor prognosis. *Gynecol Oncol*. 2003;90:331-337.
43. Yang YX, Yang AH, Yang ZJ, Wang ZR, Xia XH. Involvement of tumor suppressor in lung cancer 1 gene expression in cervical carcinogenesis. *Int J Gynecol Cancer*. 2006;16:1868-1872.
44. Backes DM, Kurman RJ, Pimenta JM, Smith JS. Systematic review of human papillomavirus prevalence in invasive penile cancer. *Cancer Causes Control*. 2009;20:449-457.
45. Smith JS, Backes DM, Hoots BE, Kurman RJ, Pimenta JM. Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors. *Obstet Gynecol*. 2009;113:917-924.
46. Grussendorf-Conen EI. Anogenital premalignant and malignant tumors (including Buschke-Lowenstein tumors). *Clin Dermatol*. 1997;15:377-388.
47. Masih AS, Stoler MH, Farrow GM, Wooldridge TN, Johansson SL. Penile verrucous carcinoma: a clinicopathologic, human papillomavirus typing and flow cytometric analysis. *Mod Pathol*. 1992;5:48-55.
48. Williams AB, Darragh TM, Vranizan K, Ochia C, Moss AR, Palefsky JM. Anal and cervical human papillomavirus infection and risk of anal and cervical epithelial abnormalities in human immunodeficiency virus-infected women. *Obstet Gynecol*. 1994;83:205-211.

49. Park IU, Palefsky JM. Evaluation and management of anal intraepithelial neoplasia in HIV-negative and HIV-positive men who have sex with men. *Curr Infect Dis Rep*. 2010;12:126-133.
50. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011;29:4294-4301.
51. Jordan RC, Lingen MW, Perez-Ordonez B, et al. Validation of methods for oropharyngeal cancer HPV status determination in U.S. cooperative group trials. *Am J Surg Pathol*. 2012;36:945-954.
52. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363:24-35.
53. Ramqvist T, Dalianis T. An epidemic of oropharyngeal squamous cell carcinoma (OSCC) due to human papillomavirus (HPV) infection and aspects of treatment and prevention. *Anticancer Res*. 2011;31:1515-1519.
54. Orth G. Epidermodysplasia verruciformis. In: Howley PM, ed. *The Papovaviridae: Volume 2. The Papillomaviruses*. New York: Plenum Press; 1987:199-243.
55. Ramoz N, Taieb A, Rueda LA, et al. Evidence for a nonallelic heterogeneity of epidermodysplasia verruciformis with two susceptibility loci mapped to chromosome regions 2p21-p24 and 17q25. *J Invest Dermatol*. 2000;114:1148-1153.
56. Ramoz N, Rueda LA, Bouadjar B, Montoya LS, Orth G, Favre M. Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. *Nat Genet*. 2002;32:579-581.
57. Leigh IM, Buchanan JA, Harwood CA, Cerio R, Storey A. Role of human papillomaviruses in cutaneous and oral manifestations of immunosuppression. *J Acquir Immune Defic Syndr*. 1999;21(suppl 1):S49-S57.
58. Padlewska K, Ramoz N, Cassonnet P, et al. Mutation and abnormal expression of the p53 gene in the viral skin carcinogenesis of epidermodysplasia verruciformis. *J Invest Dermatol*. 2001;117:935-942.
59. Iftner T, Bierfelder S, Csapo Z, Pfister H. Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. *J Virol*. 1988;62:3655-3661.
60. Orth G. Human papillomaviruses associated with epidermodysplasia verruciformis in non-melanoma skin cancers: guilty or innocent? *J Invest Dermatol*. 2005;125:xii-xiii.
61. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet*. 2007;370:59-67.
62. Feltkamp MC, de Koning MN, Bavinck JN, Ter Schegget J. Betapapillomaviruses: innocent bystanders or causes of skin cancer. *J Clin Virol*. 2008;43:353-360.
63. Jackson S, Storey A. E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. *Oncogene*. 2000;19:592-598.
64. Bouwes Bavinck JN, Neale RE, Abeni D, et al. Multicenter study of the association between betapapillomavirus infection and cutaneous squamous cell carcinoma. *Cancer Res*. 2010;70:9777-9786.
65. Proby CM, Harwood CA, Neale RE, et al. A case-control study of betapapillomavirus infection and cutaneous squamous cell carcinoma in organ transplant recipients. *Am J Transplant*. 2011;11:1498-1508.
66. Shamanin V, zur Hausen H, Lavergne D, et al. Human papillomavirus infections in nonmelanoma skin cancers from renal transplant recipients and nonimmunosuppressed patients [see comments]. *J Natl Cancer Inst*. 1996;88:802-811.
67. Weissenborn SJ, Nindl I, Purdie K, et al. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J Invest Dermatol*. 2005;125:93-97.
68. Arron ST, Ruby JG, Dybbro E, Ganem D, Derisi JL. Transcriptome sequencing demonstrates that human papillomavirus is not active in cutaneous squamous cell carcinoma. *J Invest Dermatol*. 2011;131:1745-1753.
69. Lowy DR, Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest*. 2006;116:1167-1173.
70. Schiller JT, Castellsague X, Villa L, Hildesheim A. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. *Vaccine*. 2008;26S:K53-K61.
71. Brown DR, Kjaer SK, Sigurdsson K, et al. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16-26 years. *J Infect Dis*. 2009;199:926-935.
72. Herrero R, Wacholder S, Rodriguez AC, et al. Prevention of persistent human papillomavirus infection by an HPV16/18 vaccine: a community-based randomized clinical trial in Guanacaste, Costa Rica. *Cancer Discov*. 2011;1:408-419.
73. Wheeler CM, Castellsague X, Garland SM, et al. Cross-protective efficacy of HPV-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by non-vaccine oncogenic HPV types: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial. *Lancet Oncol*. 2012;13:100-110.
74. Chaturvedi AK. Beyond cervical cancer: burden of other HPV-related cancers among men and women. *J Adolesc Health*. 2010;46:S20-S26.
75. Peres J. For cancers caused by HPV, two vaccines were just the beginning. *J Natl Cancer Inst*. 2011;103:360-362.
76. Pastrana DV, Gambhira R, Buck CB, et al. Cross-neutralization of cutaneous and mucosal papillomavirus types with anti-sera to the amino terminus of L2. *Virology*. 2005;337:365-372.
77. Roden RB, Ling M, Wu TC. Vaccination to prevent and treat cervical cancer. *Hum Pathol*. 2004;35:971-982.
78. Yuan H, Estes PA, Chen Y, et al. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. *J Virol*. 2001;75:7848-7853.
79. Burkitt D. A sarcoma involving the jaws in African children. *Br J Surg*. 1958;46:218-223.
80. Burkitt D. Determining the climatic limitations of a children's cancer common in Africa. *Br Med J*. 1962;2:1019-1023.
81. Epstein MA, Barr YM. Cultivation in vitro of human lymphoblasts from Burkitt's malignant lymphoma. *Lancet*. 1964;1:252-253.
82. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet*. 1964;1:702-703.
83. Henle G, Henle W, Diehl V. Relation of Burkitt tumor associated herpes-type virus to infectious mononucleosis. *Proc Natl Acad Sci U S A*. 1968;59:94-101.
84. Longnecker R, Kieff E, Cohen JI. Epstein-Barr virus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1898-1959.
85. Mann RB, Bernard CW. Burkitts tumor: lessons from mice, monkeys, and man. *Lancet*. 1979;2:84.
86. Moss DJ, Burrows SR, Catelino DJ, et al. A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and nonendemic regions of Papua New Guinea. *Int J Cancer*. 1983;31:727-732.
87. Manolov G, Manolova Y. Marker band in one chromosome 14 from Burkitt lymphomas. *Nature*. 1972;237:33-34.
88. Lenoir GM, Taub R. Chromosomal translocations and oncogenes in Burkitt's lymphoma. In: Goldman JM, ed. *Leukaemia and Lymphoma Research: Genetic Rearrangements in Leukaemia and Lymphoma*. London: 1986:152-172.
89. Murray RJ, Kurilla MG, Brooks JM, et al. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implication for the immune control of EBV-positive malignancies. *J Exp Med*. 1992;176:157-168.
90. zur Hausen H, Schulte-Holthausen H, Klein G, et al. EBV DNA in biopsies of Burkitt tumors and anaplastic carcinomas of the nasopharynx. *Nature*. 1970;228:1056-1058.
91. Andersson-Anvret M, Forsby N, Klein G, Henle W. Relationship between the Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: correlated nucleic acid hybridization and histopathological examination. *Int J Cancer*. 1977;20:486-494.
92. Raab-Traub N, Flynn K, Pearson G, et al. The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA. *Int J Cancer*. 1987;39:25-29.
93. Pagano JS. Epstein-Barr virus transcription in nasopharyngeal carcinoma. *J Virol*. 1983;48:580-590.
94. Weiss L, Movahed L, Warnke R, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. *N Engl J Med*. 1989;320:502-506.
95. Weiss L, Strickler J, Warnke R, Purtilo D, Sklar J. Epstein-Barr viral DNA in tissues of Hodgkin's disease. *Am J Pathol*. 1987;129:86-91.
96. Herbst H, Dallenbach F, Hummel M, et al. Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. *Proc Natl Acad Sci U S A*. 1991;88:4766-4770.

97. Herbst H, Niedobitek G, Kneba M, et al. High incidence of Epstein-Barr virus genomes in Hodgkin's disease. *Am J Pathol*. 1990;137:13-18.
98. Pallesen G, Hamilton-Dutoit S, Rose M, Young L. Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. *Lancet*. 1991;337:320-322.
99. Hamilton-Dutoit SJ, Pallesen G. A survey of Epstein-Barr virus gene expression in sporadic non-Hodgkin's lymphomas. Detection of Epstein-Barr virus in a subset of peripheral T-cell lymphomas. *Am J Pathol*. 1992;140:1315-1325.
100. Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science*. 1994;266:1865-1869.
101. Ganem D. AIDS. Viruses, cytokines and Kaposi's sarcoma. *Curr Biol*. 1995;5:469-471.
102. Ensoli B, Barillari G, Gallo RC. Pathogenesis of AIDS-associated Kaposi's sarcoma. *Hematol Oncol Clin North Am*. 1991;5:281-295.
103. Sarid R, Olsen SJ, Moore PS. Kaposi's sarcoma-associated herpesvirus: epidemiology, virology and molecular biology. *Adv Virus Res*. 1999;52:139-232.
104. Cesarman E, Moore PS, Rao PH, Inghirami G, Knowles DM, Chang Y. In vitro establishment and characterization of two AIDS-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood*. 1995;86:2708-2714.
105. Renne R, Zhong W, Herndier B, McGrath M, Kedes D, Ganem D. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in a cultured B cell lymphoma line. *Nat Med*. 1996;2:342-346.
106. Moore PS, Chang Y. Antiviral activity of tumor-suppressor pathways: clues from molecular piracy by KSHV. *Trends Genet*. 1998;14:144-150.
107. Damania B, Cesarman E. Kaposi's sarcoma-associated herpesvirus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:2080-2128.
108. Beasley RP, Lin CC, Hwang L, Chien C. Hepatocellular carcinoma and hepatitis B virus: a prospective study of 22,707 men in Taiwan. *Lancet*. 1981;2:1129-1133.
109. Evans AA, Chen G, Ross EA, Shen FM, Lin WY, London WT. Eight-year follow-up of the 90,000-person Haimen City cohort: I. Hepatocellular carcinoma mortality, risk factors, and gender differences. *Cancer Epidemiol Biomarkers Prev*. 2002;11:369-376.
110. Seeger C, Zoulim F, Mason WS. Hepadnaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:2185-2221.
111. Summers J, Mason WS. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell*. 1982;29:403-415.
112. Hansen LJ, Tennant BC, Seeger C, Ganem D. Differential activation of myc gene family members in hepatic carcinogenesis by closely related hepatitis B virus. *Mol Cell Biol*. 1993;13:659-667.
113. Koike K, Moriya K, Iino S, et al. High-level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology*. 1994;19:810-819.
114. Chisari FV, Klopchin K, Moriyama T, et al. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell*. 1989;59:1145-1156.
115. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Ann Rev Immun*. 1995;13:29-60.
116. Nakamato Y, Guidotti LG, Kuhlen CV, Fowler P, Chisari FV. Immune pathogenesis of hepatocellular carcinogenesis. *J Exp Med*. 1998;188:341-350.
117. Beasley RP. Hepatitis B virus—the major etiology of the hepatocellular carcinoma. *Cancer*. 1988;61:1942-1956.
118. Chang MH, You SL, Chen CJ, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: a 20-year follow-up study. *J Natl Cancer Inst*. 2009;101:1348-1355.
119. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int J Cancer*. 1998;75:347-354.
120. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*. 1989;244:359-362.
121. Kuo G, Choo QL, Alter HJ, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science*. 1989;244:362-364.
122. Ray SC, Bailey JR, Thomas DL. Hepatitis C virus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:795-824.
123. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A*. 1980;77:7415-7419.
124. Poiesz BJ, Ruscetti FW, Reitz MS, Kalyanaraman VS, Gallo RC. Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukemia. *Nature*. 1981;294:268-271.
125. Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in disease. *Anat Rev*. 1982;117:532.
126. Kalyanaraman VS, Sarngadharan MG, Robert-Guroff M, Miyoshi I, Golde D, Gallo RC. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science*. 1982;218:571-573.
127. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia-clinical and hematological features of 16 cases. *Blood*. 1977;50:481-492.
128. Hinuma T, Nagata K, Misoka M, et al. Adult T cell leukemia: antigen in an ATL cell line and detection of antibodies of the antigen in human sera. *Proc Natl Acad Sci U S A*. 1981;78:6476-6480.
129. Fujii M, Matsuoka M. Human T-cell leukemia virus types 1 and 2. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1474-1501.
130. Greene WC, Leonard WJ, Wano Y, et al. Trans-activator gene of HTLV-11 induces IL-2 cellular gene expression. *Science*. 1986;232:877-880.
131. Suzuki T, Hirai H, Murakami T, Yoshida M. Tax protein of HTLV-1 destabilizes the complexes of NF-kappa B and I kappa B-alpha and induces nuclear translocation of NF-kappa B for transcriptional activation. *Oncogene*. 1995;10:1199-1207.
132. Hirai H, Suzuki T, Fujisawa J, Inoue J, Yoshida M. Tax protein of human T-cell leukemia virus type I binds to the ankyrin motifs of inhibitory factor kappa B and induces nuclear translocation of transcription factor NF-kappa B proteins for transcriptional activation. *Proc Natl Acad Sci U S A*. 1994;91:3584-3588.
133. Suzuki T, Kitao S, Matsushime H, Yoshida M. HTLV-1 Tax protein interacts with cyclin-dependent kinase inhibitor p16INK4A and counteracts its inhibitory activity towards CDK4. *EMBO J*. 1996;15:1607-1614.
134. Pise-Masison CA, Brady JN. Setting the stage for transformation: HTLV-1 Tax inhibition of p53 function. *Front Biosci*. 2005;10:919-930.
135. Kawata S, Ariumi Y, Shimotohno K. p21(Waf1/Cip1/Sdi1) prevents apoptosis as well as stimulates growth in cells transformed or immortalized by human T-cell leukemia virus type 1-encoded tax. *J Virol*. 2003;77:7291-7299.
136. Kao SY, Lemoine FJ, Marriott SJ. Suppression of DNA repair by human T cell leukemia virus type 1 Tax is rescued by a functional p53 signaling pathway. *J Biol Chem*. 2000;275:35926-35931.
137. Liu B, Liang MH, Kuo YL, et al. Human T-lymphotropic virus type 1 oncoprotein tax promotes unscheduled degradation of Pds1p/securin and Clb2p/cyclin B1 and causes chromosomal instability. *Mol Cell Biol*. 2003;23:5269-5281.
138. Satou Y, Yasunaga J, Yoshida M, Matsuoka M. HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. *Proc Natl Acad Sci U S A*. 2006;103:720-725.
139. McClure MO, Weiss RA. Human immunodeficiency virus and related viruses. *Curr Top AIDS*. 1987;1:95-117.
140. Pinching A, Weiss RA. AIDS and the spectrum of HTLV-III/LAV infection. *Int Rev Exp Pathol*. 1986;28:1-44.
141. Poulin DL, DeCaprio JA. Is there a role for SV40 in human cancer? *J Clin Oncol*. 2006;24:4356-4365.
142. Shah KV. SV40 and human cancer: a review of recent data. *Int J Cancer*. 2007;120:215-223.
143. Lopez-Rios F, Illei PB, Rusch V, Ladanyi M. Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids. *Lancet*. 2004;364:1157-1166.
144. Manfredi JJ, Dong J, Liu WJ, et al. Evidence against a role for SV40 in human mesothelioma. *Cancer Res*. 2005;65:2602-2609.

145. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science*. 2008;319:1096-1100.
146. Toker C. Trabecular carcinoma of the skin. *Arch Dermatol*. 1972;105:107-110.
147. Tolstov YL, Pastrana DV, Feng H, et al. Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. *Int J Cancer*. 2009;125:1250-1256.
148. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB. Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. *Cell Host Microbe*. 2010;7:509-515.
149. DeCaprio JA, Imperiale MJ, Major EO. Polyomaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2013:1633-1661.
150. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet*. 1984;1:1311-1315.
151. Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Med J Aust*. 1985;142:436-439.
152. Tomb JF, White O, Kerlavage AR, et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*. 1997;388:539-547.
153. Hatakeyama M. *Helicobacter pylori* CagA—a bacterial intruder conspiring gastric carcinogenesis. *Int J Cancer*. 2006;119:1217-1223.
154. Tsuji S, Kawai N, Tsujii M, Kawano S, Hori M. Review article: inflammation-related promotion of gastrointestinal carcinogenesis—a perigenetic pathway. *Aliment Pharmacol Ther*. 2003;18(suppl 1):82-89.
155. zur Hausen H. *Infections Causing Human Cancer*. Weinheim, Germany: Wiley-VCH; 2006.

Erika L. Abel and John DiGiovanni

7

Environmental Carcinogenesis

Introduction to Cancer and the Environment

Environment, Genetics, and Cancer

Overall human cancer risk is determined by complex interactions between host genetics and environmental exposures. On exposure to a cancer-causing agent, a cascade of events is set into motion that converts normal cells into cancer cells. This process is referred to as *carcinogenesis*, and cancer-causing agents are referred to as *carcinogens*. Hundreds of confirmed and suspected environmental carcinogens have been identified. Environmental factors are generally believed to account for a significant portion of cancer mortality worldwide. In the context of the current chapter, we refer to the *environment* as any substance or agent that is normally present outside of the human body and that interacts with the human body to increase cancer risk. Genetically controlled host factors also contribute to cancer risk, primarily through modulation of responses to environmental agents. Understanding the causes of cancer and the underlying mechanisms that lead to cancer development provides a rational basis for developing prevention strategies. In this chapter, we discuss the major known environmental causes of cancer and, where applicable, underlying mechanisms. In addition, where known, significant gene-environment interactions are highlighted.

History of Chemicals and Cancer

The environmental contribution to chronic diseases such as cancer has been recognized for centuries.[1,2] In 1775, Dr. Percival Pott observed that chimney sweeps experienced an increased incidence of scrotal cancer, which was attributed to frequent and heavy exposure to soot. A century later, an excess of skin cancers was reported in coal tar workers in Germany and related to their occupational exposure. In the early 20th century, these observations were experimentally validated by Yamagiwa and Ichikawa, who demonstrated that multiple topical applications of coal tar to rabbit ears induced skin carcinomas. These studies were the first to demonstrate that a complex mixture was capable of inducing cancer. Sir Ernest Kennaway and others furthered these studies in the 1920s and 1930s. The group fractionated coal tar with the goal of isolating the principal carcinogenic agent. Fractions were screened for a characteristic blue-violet fluorescence spectrum, which was highly correlated with carcinogenic potency.[3] Ultimately, benzo[*a*]pyrene (B[*a*]P), which is composed entirely of carbon and hydrogen (Figure 7-1), was identified, synthesized, and shown to be a potent carcinogen in animal models. Collectively, these important early studies indelibly transformed the study of environmental carcinogenesis. In isolating a compound from coal tar that could induce cancer in animals, an occupational carcinogen exposure was linked to cancer incidence, and the utility of animal models of carcinogenicity in the interpretation of human epidemiologic associations was established.

Kennaway's studies in the early 20th century immediately preceded major progress in understanding the biochemical nature of genetic material and cellular replication. Beginning shortly after the discovery of benzo[*a*]pyrene, Avery and colleagues pursued experiments to reveal the identity of the transforming material of pathogenic *Streptococcus pneumoniae*. Results of their analyses showed that nucleic acids carried genetic information, although their findings were not widely accepted until the 1950s when Hershey and Chase published data demonstrating that DNA is the genetic material of viruses. Shortly thereafter, work with mutagenic mustard gas suggested that DNA was the target of carcinogens, and data published in the early 1960s revealed that mutagens covalently modify DNA.[1] Further, the carcinogenic potency of a series of hydrocarbons was positively correlated with the extent of their reaction with DNA. These and other studies provided compelling evidence for DNA as a target of carcinogens and gene mutation as a major mechanism whereby agents induce cancer. With this foundational knowledge, the list of known carcinogens has grown rapidly, along with a greater understanding of the mechanisms of cancer development associated with these agents.

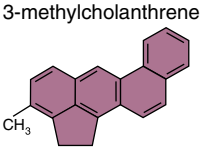
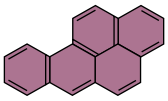
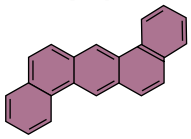
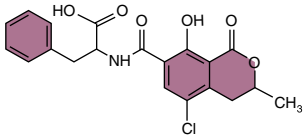
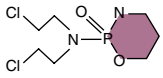
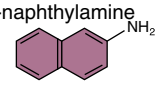
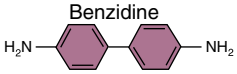
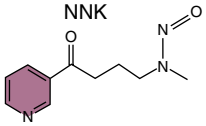
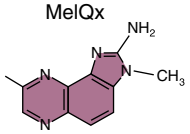
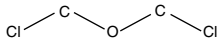
CARCINOGEN CLASS	REPRESENTATIVE EXAMPLE
PAHs	3-methylcholanthrene  Benzo[*a*]pyrene  Dibenzo[*a*,*h*]anthracene 
Solvents	Benzene 
Mycotoxins	Ochratoxin A 
Alkyl halides	Vinyl chloride  Cyclophosphamide 
Aromatic amines	2-naphthylamine  Benzidine 
N-nitrosamines	NNK 
Heterocyclic amines	MeIQx 
Alkylating agents	bis(chloromethyl)ether 

Figure 7-1 Chemical structures of selected carcinogens.

Causes of Cancer

Epidemiology and Causal Criteria

The landmark findings of Kennaway, Avery, Hershey, Chase, and others in the early part of the 1900s guided a period of rapid advancement in the laboratory concerning the molecular basis of cancer. In contrast, the discovery of specific human carcinogens has been largely guided by epidemiologic studies of cancer incidence. The study of worldwide cancer incidence patterns, including analysis of cancer risk among migrant populations, has confirmed the critical role of environment in determining cancer risk. Studies of exposure cohorts and observational studies of cancer incidence have been especially crucial in the identification of the biologic, physical, and chemical agents capable of causing cancer. Similarly, epidemiologic studies have revealed numerous lifestyle choices and socioeconomic factors associated with increased risk of cancer.

Cancer risk is known to vary extensively worldwide.[4] For instance, liver cancer risk varies 20- to 40-fold internationally; the incidence is highest in eastern Asia and lowest in northern Europe and Central America. Prostate cancer rates are high in the United States, Canada, and Scandinavia, especially in comparison with the rates in China and other Asian countries. Similarly, breast cancer risk has historically been higher in the United States and European countries than in Asia, Africa, and South America. These observations suggest that (1) genetic differences among ethnic groups alter cancer risk and/or (2) differences in environmental exposures among geographic locations affect the risk of developing cancer.

Capitalizing on known ethnic variation in cancer rates, analysis of cancer risk in migrant populations has been undertaken and has yielded important information concerning the relative contribution of environment versus genetics in cancer etiology. In these studies, the rate of cancer in migrant cohorts is compared with the rate of cancer among people of the same ethnicity living in the country of origin and to the cancer rate of people in the destination population. For example, breast cancer incidence among Asian immigrants to the United States has been compared with that of women still living in their country or region of origin.[5] The breast cancer risk of Asian American women born in the East has been shown to rise with increasing number of years lived in the West. Ultimately, the risk of breast cancer among Asian American women approaches that of U.S.-born White women and is significantly higher than that of Asian women still living in the country of origin. Numerous studies of this kind demonstrate that even while in the first generation following relocation, immigrant populations assume a pattern of cancer risk in common with native populations rather

than with populations in their country of origin. These studies imply that environmental factors play a significant role in determining cancer risk. Similarly, studies of cancer risk in twins have suggested the importance of environmental factors in determining overall cancer risk.

Recent population-based evidence further underscores the overall importance of environmental factors in determining cancer risk. Cancers that were once associated with affluence and/or the Western lifestyle are on the rise in less developed countries. Rates of colon, breast, and lung cancers in developing countries have increased as their economies have transitioned.[4,6] Multiple factors likely contribute to this trend, including non-genetically controlled influences such as tobacco use, diet, and physical activity.

In addition to population-based evidence, case-control and cohort studies have been used to identify specific environmental agents and factors that are now considered to be human carcinogens. To assess the likelihood that a particular environmental exposure is causally linked to cancer, epidemiologic data are interpreted in the context of mechanistic data and other considerations. The strength of evidence for a causal role in cancer development is evaluated using criteria developed as a modification of Bradford-Hill's criteria (1965) for assessment of evidence of causation[7]:

1. Strength of Association: Large-magnitude effects on cancer risk are less likely than small-magnitude effects to be due to chance.
2. Temporal Relationship: To be causal, the environmental exposure must have happened in advance of the appearance of cancer.
3. Biologic Plausibility: Relationships that can be supported by laboratory evidence or a plausible mechanistic hypothesis are more likely to be causal relationships.
4. Dose-Response Relationship: Studies that demonstrate a gradient in disease outcome whenever a gradient in exposure has occurred provide stronger support for a causal relationship than those studies that do not demonstrate a positive correlation between dose and response.
5. Consistency: The most probable causal relationships are consistently demonstrated in multiple studies of the exposure-disease relationship.

Using these criteria, numerous cancer-causing agents and/or risk factors have been identified for further characterization.

Known Cancer Risk Factors

In a landmark paper published in 1981, Doll and Peto summarized available epidemiologic data to estimate the percentage of U.S. cancer deaths attributable to a variety of environmental and lifestyle influences. Their analyses suggested that as many as 60% of all cancer deaths could be attributed to two environmental factors: diet and tobacco use.[8] More than 30 years later, these estimates appear to remain valid; diet and tobacco use continue to be primary determinants of cancer mortality. Additional factors cited by multiple investigators and regulatory agencies as contributing to cancer risk include occupation, radiation, alcohol, pollution, infections, medications, and reproductive and socioeconomic factors.

Smoking

Tobacco use remains the single most important and avoidable factor in determining cancer risk.[9,10] Smoking is estimated to contribute to at least 30% of all cancer deaths. Lung, bladder, esophageal, pancreatic, uterine, oral, and nasal cavity cancers, among others, have all been associated with tobacco use. Approximately 90% of all lung cancer deaths can be attributed to smoking. Lung cancer risk is greatest for persons who begin smoking at an early age and continue smoking for many years, and the risk of tobacco smoke–induced lung cancer is directly proportional to the dose inhaled. Tobacco smoke is a complex mixture of chemicals, 55 of which are known or suspected human carcinogens (Table 7-1). On absorption in the lungs, these agents may act locally or at distal sites to (1) induce DNA damage and (2) alter cellular growth and proliferation. A synergistic effect has been noted in the case

Table 7-1 Carcinogens in Tobacco Smoke

Carcinogen Class	No. of Compounds	Example Compound
Polycyclic aromatic hydrocarbons	10	Benzo[*a*]pyrene 5-Methylchrysene Dibenz[*a*,*h*]anthracene
Aza-arenes	3	Dibenz[*a*,*h*]acridine
N-nitrosamines	7	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) *N*-Nitrosodiethylamine
Aromatic amines	3	4-Aminobiphenyl
Heterocyclic amines	8	2-Amino-3-methylimidazo[*4,5-f*]quinoline
Aldehydes	2	Formaldehyde
Miscellaneous organic compounds	15	1,3-Butadiene Ethyl carbamate
Inorganic compounds	7	Nickel Chromium Cadmium Arsenic
Total	55	

Adapted from Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst* 1999;91:1194.

of combined tobacco use and heavy alcohol use. Despite antitobacco sentiment, approximately one fifth of U.S. citizens are still smokers, and smoking rates in countries such as China remain high; therefore, smoking-induced cancers are likely to continue to be prevalent worldwide.

Diet

The effects of diet on cancer risk have been attributed both to dietary chemical constituents and to overall energy consumption. As many as 14% to 30% of cancer deaths have been attributed to overweight and obesity. Overweight and obesity, as defined by the ratio of weight to height known as *body mass index* (BMI), are prevalent at epidemic proportions in the United States and other developed countries. Overweight and obesity have been associated with elevated risk of cancers of the colon, breast, endometrium, kidney, liver, pancreas, gallbladder, ovary, cervix, rectum, and esophagus as well as risk of non-Hodgkin's lymphoma and multiple myeloma. In addition, animal studies have consistently demonstrated that restricting calorie intake can significantly reduce cancer risk, whereas inducing obesity can significantly elevate cancer risk. Despite these findings, a complete understanding of the mechanistic basis for the effect of dietary energy balance status on cancer formation is not conclusively known.[11] Elevated steroid hormone production in adipose tissue has been proposed as the basis for obesity-induced endometrial and breast cancers; adipose-derived leptin, adiponectin, and proinflammatory molecules may affect cancer development more broadly. Recent studies have suggested that alterations in circulating insulin-like growth factor 1 (IGF-1) levels may account for some of the effects of altered dietary energy balance status on cancer risk.[12]

In addition to excess calorie intake, certain dietary constituents may affect cancer risk.[13] In the United States, cancer risk due to food additives is presumed to be quite low because the U.S. Food and Drug Administration (FDA) strictly regulates food additive use. In 1958, an amendment to the Food, Drugs, and Cosmetic Act of 1958, referred to as the *Delaney Clause*, was approved and stated that "the Secretary (of the FDA) shall not approve for use in food any chemical additive found to induce cancer in man, or, after tests, found to induce cancer in animals." Presumably, therefore, cancer risk due to food additive consumption is quite low. Nonetheless, inadvertent food contaminants such as the plasticizer bisphenol A remain a source of concern. Bisphenol A is a weak endocrine-disrupting agent that has been associated with a variety of health effects including increased cancer risk. Fungal toxins such as aflatoxins are food contaminants resulting from mold growth on foodstuffs. Several of these toxins have been shown to be extremely potent mutagens and in some cases potent carcinogens (e.g., aflatoxin B_1 [AFB_1]). Red meat consumption has been associated with elevated colorectal cancer risk, possibly due in part to the carcinogenic nitrosamine and heterocyclic amine content of preserved or heat-treated meats.

Although examples of carcinogenic dietary constituents can be identified, a possibly greater dietary determinant of cancer risk is consumption of anticarcinogenic fruits and vegetables. Consumption of fruits and vegetables has consistently been linked to reduced cancer risk for a variety of cancer types. Fruits and vegetables contain numerous antioxidant compounds, which may guard against oxidative DNA damage or other forms of carcinogenic assault. In fact, tea phenols such as epigallocatechin-3-gallate (EGCG), the turmeric component curcumin, grape-derived resveratrol, and lycopene from tomatoes are all proposed cancer preventive agents.[14] On the other hand, excess consumption of herbal health supplements is an emerging dietary concern due to their widespread use in the absence of proper validation or safety assessment. As an example, renal failure was noted in women who consumed weight-reducing Chinese herbal pills. The pills were inadvertently substituted with a nephrotoxic herb, *Aristolochia fangchi*, containing aristolochic acids. Aristolochic acids are mutagenic and carcinogenic, and a high rate of urothelial carcinoma was noted in the population of women who consumed these pills.[15]

Occupation

Many carcinogens have been identified at the cost of human exposure and cancer incidence that occurred as a result of industrialization. Human epidemiologic studies highlight the potency of chemical and physical carcinogens and how lack of understanding leads to lack of preparation and protection.[16-18] In the 1800s, high incidence of bladder cancer among workers in the aniline dye industry was recognized. Later, evidence was reported demonstrating that 2-napthylamine and benzidine were two carcinogenic agents responsible for this unusual cancer incidence. Also during the early 1900s, nearly 5000 workers were hired to apply luminous radium-containing paint to watch and instrument dials. Because of their occupational radiation exposure and a lack of precautionary practices, a large excess of bone cancers was noted among this cohort. Thousands of workers were exposed to vinyl chloride before its ability to induce angiosarcoma of the liver was recognized. Since the 1970s, strict workplace regulations and protective measures in the United States have largely prevented such dramatic incidents. The Occupational Safety and Health Administration (OSHA) was signed into existence in 1970 by the U.S. government with the goal of ensuring worker safety and health by improving the workplace environment. OSHA sets the legal limit for worker exposure to hazardous compounds in the United States. These limits are referred to as *permissible exposure limits* (PELs). PELs have been issued for approximately

Table 7-2 Environmental Carcinogens Associated with Occupation

Occupation	Carcinogen Exposure	Associated Cancer Type
Iron and steel founding	PAH, chromium, nickel, formaldehyde	Lung
Copper mining and smelting	Arsenic	Skin, bronchus, liver
Underground mining	Radon (ionizing radiation)	Lung
Aluminum production	PAH	Lung
Coke production	PAH	Lung, kidney
Painting	Chromium, solvents	Lung
Furniture and cabinet making	Wood dust	Nasal sinus
Boot and shoe manufacture	Leather dust, benzene	Nasal sinus, leukemia
Rubber industry	Aromatic amines, solvents	Bladder, leukemia
Nickel refining	Nickel	Nasal sinus, bronchus
Vinyl chloride manufacture	Vinyl chloride	Liver
Dye and textile production	Benzidine-based dyes	Bladder

PAH, Polycyclic aromatic hydrocarbons.

500 chemicals, a portion of which are known or suspected carcinogens. Also created in 1970, the Environmental Protection Agency (EPA) is charged with protecting human health and the environment. In addition to other roles, the EPA regulates the release of industrial pollution, including carcinogens. Before these institutions were in place, employment in a wide variety of settings was linked to elevated risk of numerous cancers (Table 7-2).

Despite regulatory measures, occupational exposure to carcinogens continues. In the U.S. President's Cancer Panel Report of 2008-2009,[19] members highlighted 14 types of environmental contaminants from industrial, manufacturing, and agricultural sources (polyhalogenated biphenyls, asbestos, chromium, perchloroethylene/trichloroethylene, particulate matter, mercury, formaldehyde, endocrine-disrupting chemicals, atrazine, DDT, nitrogen fertilizers, phosphate fertilizers, and veterinary pharmaceuticals) due to their cancer-causing potential and high probability of human exposure. The group estimated that millions of workers continue to be exposed to high levels of these and other agents each year. The families of exposed workers also experience higher than average exposure due to home contamination and may be at elevated cancer risk. As examples, chromium used in leather tanning, manufacture of dyes and pigments, wood preservation, and chrome plating is an established risk factor for lung cancer. Perchloroethylene, heavily used in dry-cleaning businesses, is classified as a probable carcinogen by the International Agency for Research on Cancer (IARC), and formaldehyde (a group 1 human carcinogen) is a synthetic starting material in manufacturing and a widely used disinfectant and preservative.

Causes of Cancer by Organ Site

Although an extensive list of known human carcinogens has been collected, the cause of many common cancers is still unknown. As shown in Table 7-3, gastric, liver, and cervical cancers are each clearly linked with biologic carcinogens: *Helicobacter pylori*, hepatitis B virus (HBV), and human papillomavirus (HPV), respectively. The vast majority of lung cancer cases can be linked to tobacco use, and mesothelioma incidence is strongly correlated with exposure to asbestos. In contrast, the causes of most brain, pancreas, and prostate cancers remain largely unknown. For many other cancer types such as bone cancers, relatively rare exposures have been causally linked to incidence, yet the associated attributable risk is quite low. The remainder of cases continues to be largely unexplained. In general, linking particular cancers to specific exposure events can be problematic, and further work is necessary to uncover the primary causes of a significant number of cancers. Limiting factors include the inability to accurately estimate exposure dose and duration and a lack of understanding of combinatorial effects in multi-exposure events and finally lack of adequate biomarkers of exposure.

Classes and Types of Carcinogens

Carcinogen Evaluation and Classification

The U.S. National Toxicology Program (NTP), the World Health Organization's International Agency for Research on Cancer (IARC), the U.S. EPA, and other agencies characterize and report the carcinogenicity of environmental agents and other factors (including drugs). Each entity independently evaluates the available evidence to rate the cancer-causing potential of a chemical, chemical mixture, occupational exposure, physical agent, biologic agent, or lifestyle factor. The most frequently referenced database is the IARC *Monographs on the Evaluation of Carcinogenic Risks to Humans*. IARC defines carcinogens as agents "capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity." Agents are selected for evaluation on the basis of two factors: (1) evidence of potential carcinogenicity and (2) known exposure of humans. During the scientific review and evaluation of potential carcinogens, a working group is

Table 7-3 Exposures Associated with Human Cancers, as Identified by the IARC (Partial Listing)

Cancer Site	Carcinogenic Agents with Sufficient Evidence in Humans	Agents with Limited Evidence in Humans
Oral cavity	Alcohol, betel quid, HPV, tobacco smoking, smokeless tobacco	Solar radiation
Stomach	*Helicobacter pylori*, rubber production industry, tobacco smoking, x-rays, gamma radiation	Asbestos, Epstein-Barr virus, lead, nitrate, nitrite, pickled vegetables, salted fish
Colon and rectum	Alcohol, tobacco smoking, radiation	Asbestos, *Schistosoma japonicum*
Liver and bile duct	Aflatoxins, alcohol, *Clonorchis sinensis*, estrogen-progestin contraceptives, HBV, HCV, *Opisthorchis viverrini,* plutonium, thorium-232, vinyl chloride	Androgenic steroids, arsenic, betel quid, HIV, polychlorinated biphenyls, *Schistosoma japonicum*, trichloroethylene, x-rays, gamma radiation
Pancreas	Tobacco smoking, smokeless tobacco	Alcohol, thorium-232, x-rays, gamma radiation, radioiodines
Lung	Tobacco smoking, aluminum production, arsenic, asbestos, beryllium, bis (chloromethyl) ether, chloromethyl methyl ether, cadmium, chromium, coal combustion and coal tar pitch, coke production, hematite mining, iron and steel founding, MOPP, nickel, painting, plutonium, radon, rubber production, silica dust, soot, sulfur mustard, x-rays, gamma radiation	Acid mists, manufacture of glass, indoor emissions from household combustion, carbon electrode manufacture, chlorinated toluenes and benzoyl chloride, cobalt metal with tungsten carbide, creosotes, engine exhaust, insecticides, dioxin, printing processes, welding fumes
Skin—melanoma	Solar radiation, UV-emitting tanning devices	
Other skin cancers	Arsenic, azathiopurine, coal tar pitch, coal tar distillation, cyclosporine, methoxsalen plus UVA, mineral oils, shale oils, solar radiation, soot, x-rays, gamma radiation	Creosotes, HIV, HPV, nitrogen mustard, petroleum refining, UV-emitting tanning devices
Mesothelioma	Asbestos, erionite, painting	
Breast	Alcohol, diethylstilbestrol, estrogen-progesterone contraceptive and menopausal therapy, x-rays, gamma radiation	Estrogen menopausal therapy, ethylene oxide, shift work resulting in circadian disruption, tobacco smoking
Uterine cervix	Diethylstilbestrol (exposure in utero), estrogen-progestogen contraception, HIV, HPV, tobacco smoking	Tetrachloroethylene
Ovary	Asbestos, estrogen menopausal therapy, tobacco smoking	Talc-based body powder, x-rays, gamma radiation
Prostate		Androgenic steroids, arsenic, cadmium, rubber production industry, thorium-232, x-rays, gamma radiation, diethylstilbestrol (exposure in utero)
Kidney	Tobacco smoking, x-rays, gamma radiation	Arsenic, cadmium, printing processes
Urinary Bladder	Aluminum production, 4-aminobiphenyl, arsenic, auramine production, benzidine, chlornaphazine, cyclophosphamide, magenta production, 2-naphthylamine, painting, rubber production, *Schistosoma haematobium,* tobacco smoking, toluidine, x-rays, gamma radiation	Coal tar pitch, coffee, dry cleaning, engine exhaust, printing processes, occupational exposures in hair dressing and barbering, soot, textile manufacturing
Brain	X radiation, gamma radiation	
Leukemia and/or lymphoma	Azathiopurine, benzene, busulfan, 1,3-butadiene, chlorambucil, cyclophosphamide, cyclosporine, Epstein-Barr virus, etoposide with cisplatin and bleomycin, fission products, formaldehyde, *Helicobacter pylori*, HCV, HIV, human T-cell lymphotropic virus type 1, Kaposi's sarcoma herpesvirus, melphalan, MOPP, phosphorus-32, rubber production, semustine, thiotepa, thorium-232, tobacco smoking, treosulfan, X radiation, gamma radiation	Bischloroethyl nitrosourea, chloramphenicol, ethylene oxide, etoposide, HBV, magnetic fields, mitoxantrone, nitrogen mustard, painting, petroleum refining, polychlorophenols, radioiodines, radon-222, styrene, teniposide, tetrachloroethylene, trichloroethylene, dioxin, tobacco smoking (childhood leukemia in smokers' children)

Adapted from Cogliano et al. Preventable exposures associated with human cancers. *J Natl Cancer Inst* 2011;103:1835.
HBV, Hepatitis B virus; *HCV,* hepatitis C virus; *HIV,* human immunodeficiency virus; *HPV,* human papillomavirus; *IARC,* International Agency for Research on Cancer; *MOPP,* mustargen-oncovin-procarbazine-prednisone chemotherapy; *UVA,* ultraviolet A light.

formed and charged with summarizing available data concerning anticipated exposure levels, human epidemiologic data, and studies of cancer-producing capacity in animals. Although the goal of the IARC *Monographs* has been to identify carcinogens regardless of an explanatory mechanism, information on mechanisms can also be used as supporting data. All agents evaluated by IARC are classified into one of five categories as shown in Table 7-4. As of the most recent report, 108 agents, groups of agents, or exposure scenarios are listed as "Carcinogenic to Humans" (a partial listing is shown in Table 7-5). An additional 64 are listed as "Probably Carcinogenic to Humans." These agents are extremely diverse in structure, potency, and mechanism.

Table 7-4 IARC Classification of Suspected Carcinogenic Agents

Group 1: Carcinogenic to humans: Sufficient evidence of carcinogenicity in humans exists or sufficient evidence of carcinogenicity in animals is supported by strong evidence of a relevant mechanism of carcinogenicity in humans.
Group 2A: Probably carcinogenic to humans: Limited evidence of carcinogenicity in humans exists but sufficient evidence of carcinogenicity in animals has been demonstrated. Alternatively, inadequate evidence in humans with sufficient evidence in animals may be supported by strong evidence that a similar mechanism of carcinogenicity would occur in humans.
Group 2B: Possibly carcinogenic to humans: Limited evidence of carcinogenicity in humans exists but inadequate evidence in experimental animals. Alternatively, this classification can be used for agents for which there are inadequate data in humans but sufficient evidence in animals or strong mechanistic data.
Group 3: Unclassifiable as to carcinogenicity in humans: Inadequate evidence in humans and animals exists. Alternatively, sufficient evidence of carcinogenicity may exist in animals but strong mechanistic data predict a lack of carcinogenicity in humans.
Group 4: Probably not carcinogenic to humans: Evidence suggesting a lack of carcinogenicity in humans and animals exists.

IARC, International Agency for Research on Cancer.

Types of Carcinogens

Carcinogens can be grouped into one of three categories according to their composition: (1) physical carcinogens, (2) biologic carcinogens, and (3) chemical carcinogens. The term *physical carcinogen* encompasses multiple types of radiation (e.g., ultraviolet [UV] and ionizing radiation). *Biologic carcinogens* refer to viral and bacterial infections that have been associated with cancer development (e.g., human papillomavirus [HPV] and hepatitis B virus [HBV]). Most carcinogens can be categorized as chemical carcinogens. As examples, heavy metals, organic combustion products (e.g., B[*a*]P), hormones, and fibers (e.g., asbestos) are considered to be chemical carcinogens. Note that in the discussion that follows, only selected carcinogens that are known to be carcinogenic in humans are described (see Table 7-5). For a more comprehensive listing of carcinogenic agents, including those listed in other IARC categories, refer to the WHO IARC monograph database (http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php) and additional references.[20,21]

Physical Carcinogens

Examples of physical carcinogens include UV and ionizing radiation. *Radiation* refers to flow of energy-bearing particles; *ionizing radiation* refers to radiation that is of sufficiently high energy to remove an electron from an atom or molecule with which it collides. Exposure to ionizing radiation of various forms has been shown to cause multiple types of cancers. In addition, solar radiation is of sufficient energy to elicit photochemical damage to the skin, ultimately leading to cancer formation.

Table 7-5 Selected IARC Known Human Carcinogens

4-Aminobiphenyl	Hepatitis B virus
Arsenic	Hepatitis C virus
Asbestos	Human immunodeficiency virus type 1
Azathioprine	Human papillomavirus
Benzene	Human T-cell lymphotropic virus
Benzidine	Melphalan
Benzo[*a*]pyrene	8-Methoxypsoralen
Beryllium	Mustard gas
N,*N*-Bis(2-chloroethyl)-2-naphthylamine	2-Naphthylamine
Bis(chloromethyl)ether	Nickel compounds
Chloromethyl methyl ether	*N*′-Nitrosonornicotine (NNN)
1,4-Butanediol dimethanesulfonate	Phosphorus-32
Cadmium	Plutonium-239
Chlorambucil	Radioiodines
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea	Radium-224
Chromium[VI]	Radium-226
Cyclosporine	Radium-228
Cyclophosphamide	Radon-222
Diethylstilbestrol	Silica
Epstein-Barr virus	Solar radiation
Erionite	Talc-containing asbestiform fibers
Estrogen-progestogen menopausal therapy	Tamoxifen
Estrogen-progestogen oral contraceptives	2,3,7,8-Tetrachlorodibenzo-*para*-dioxin
Estrogen therapy	Thiotepa
Ethylene oxide	Treosulfan
Etoposide	Vinyl chloride
Formaldehyde	X- and gamma (γ)-radiation
Gallium arsenide	Aflatoxins
Helicobacter pylori	Soots Tobacco Wood dust

IARC, International Agency for Research on Cancer.

The incidence of skin cancers such as melanoma, basal-cell carcinoma, and squamous-cell carcinoma has risen dramatically in recent years.[22] The risk of developing skin cancer is highest in equatorial regions and correlates with the number of blistering sunburns encountered during childhood. Correlative studies such as these, in addition to mechanistic studies at the cellular and organismal levels,

indicate that most skin cancers arise because of exposure to solar radiation. In particular, UV radiation in the 100- to 400-nm range appears to be causative. The health effects of UV radiation vary according to wavelength. Consequently, UV radiation is examined in three regions of wavelength: UVA, 315 to 400 nm; UVB, 280 to 315 nm; UVC, 100 to 280 nm. In contrast to UVC radiation, UVB and UVA can bypass the earth's atmosphere, including stratospheric ozone; therefore, UVA and UVB are believed to contribute to a much higher attributable risk of cutaneous carcinogenesis than UVC. Moderate UVB exposure results in an erythema response, and UVB is well absorbed by cellular molecules such as DNA, melanin, amino acids, carotene, and urocanic acids.[23,24] UVB is more potent in inducing skin tumors in hairless mice than UVA. However, exposure to UV light of any wavelength results in DNA damage and mutation in in vitro models, and UVA also induces tumors in hairless mice. For this reason, excess exposure to any wavelength of UV light is considered unsafe, and tanning beds have been placed on the IARC's list of human carcinogens.

For UV radiation to produce an adverse reaction in skin, photon energy must be absorbed by the target biomolecules such as DNA. Although melanin produced by resident melanocytes is a critical UV radiation absorption filter, unfiltered photons may generate oxidative stress and/or damage DNA. UV irradiation of DNA results in the formation of pyrimidine dimers and other photodamage such as DNA strand breaks and pyrimidine-pyrimidone photoproducts.[25] When these lesions are not repaired, DNA mutations can result. The hallmark UVB radiation-induced mutations are C→T or CC→TT transitions. Target genes for solar radiation–induced mutations include but are not limited to *TP53* (squamous-cell carcinomas [SCCs], basal-cell carcinomas [BCCs], melanoma), *CDKN2A* (melanoma), *BRAF* (melanoma), *NEDD9* (melanoma), and *PTCH* (BCCs, possibly SCCs). UV irradiation of skin keratinocytes also alters numerous cell signaling pathways such as growth arrest and DNA damage-response (i.e., *p53*, *GADD45*, mismatch repair genes), apoptotic (i.e., bcl-2, fas), and mitogenic (i.e., ras, ERK) signaling pathways.[26]

In addition to solar radiation, ionizing radiation in the form of x-rays, nuclear fallout, and therapeutic irradiation as well as energy deposition from radon gas also contribute to the incidence of human cancers. Epidemiologic studies of radiation workers and atom bomb survivors of Hiroshima and Nagasaki as well as the use of animal models have led to the characterization of ionizing radiation as a "universal carcinogen."[27] Ionizing radiation can induce tumors in most tissues and in most species examined because of its unique ability to penetrate tissues and induce DNA damage via energy deposition.[28]

Radon-222 is a radioactive gas that is produced by radioactive decay of uranium-238, which is found ubiquitously in soil, rock, and groundwater. Concern over accumulation of radon in indoor air, especially in underground spaces, has led to study of the health effects of inhaled radon. Radon decay results in the release of alpha particles (two protons and two neutrons), which do not deeply penetrate tissues but possess the capacity to damage DNA in areas of contact. Inhalation of radon has been associated with lung cancer incidence due to exposure of the bronchial epithelium to decay products.[29] Uranium miners have been shown to succumb to lung cancer at a much higher rate than the general population because of their exposure to radon in underground air supplies. At the reduced exposure level detected in homes, radon carcinogenic potential is low, although not insignificant. WHO officials consider radon to be "the second most important cause of lung cancer second to tobacco in many countries" (http://www.who.int/phe/radiation/backgrounder_radon/en/index.html).

Biologic Carcinogens

Biologic carcinogens also play an important role in human carcinogenesis. Approximately 20% of human cancers are associated with infectious agents including bacteria, parasites, and viruses. These are discussed in more detail in Chapter 6 and are not discussed further in this chapter.

Chemical Carcinogens

Chemical carcinogens can be classified into one of four groups according to their chemical nature: organic carcinogens, inorganic carcinogens, fibers, and hormones. The first experimental confirmation of the existence of organic chemical carcinogens came in 1915, when Yamagiwa and Ichikawa demonstrated that multiple applications of coal tar could induce skin tumors on the ears of rabbits.[30] It was later shown that the active carcinogenic agent was composed entirely of carbon and hydrogen. Since that time, numerous carbon-based carcinogens have been identified in studies using experimental animals and in epidemiologic studies of human populations. These organic compounds range from industrially produced and utilized solvents, to naturally occurring but chemically complex combustion products and mycotoxins, to simple alkyl halides such as vinyl chloride (see Figure 7-1).

Organic Carcinogens

Benzene

Benzene is a widely used solvent and is present in gasoline, automobile emissions, and cigarette smoke. Historically, high-level exposure to benzene was commonplace, and, in general, benzene exposure has been the cause of great concern due to its carcinogenic properties. Exposure to benzene

occurs in industrial settings such as in rubber production, chemical plants, oil refineries, and shoe manufacturing. Because benzene is a volatile aromatic solvent, inhalation exposures predominate.[31]

The carcinogenic properties of benzene have long been recognized; an increased risk of leukemia has been shown in workers exposed to high levels of benzene. Benzene exposure is associated with myelodysplastic syndromes. In addition, the strongest associations of benzene and cancer risk are found with risk of acute myeloid leukemia and non-Hodgkin's lymphoma. Benzene is a recognized clastogen and induces oxidative stress upon metabolic activation. Along with mutagenic effects, benzene is believed to alter cell-signaling pathways that control hematopoiesis in hematopoietic stem cells.[32] Workplace exposure restrictions have reduced human exposure to high levels of benzene. Current research is aimed at assessing risk associated with chronic low-level exposure scenarios.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of intensively studied organic compounds including benzo[*a*]pyrene. Many PAHs can be metabolically activated to become highly reactive, electrophilic mutagens. PAHs are converted to "bay region" diol epoxides as depicted in Figure 7-2. These diol epoxides covalently bind to DNA, forming a DNA adduct, and their overall reactivity is predictive of their carcinogenic potency.[2,33] For example, benzo[*a*]pyrene diol epoxide reacts extensively with the exocyclic amino group of guanine to produce mutagenic DNA adducts (Figure 7-3, and see section entitled Initiation and Mutational Theory of Carcinogenesis). In addition, certain PAH metabolites may act synergistically with bay region diol epoxide metabolites to promote tumor formation in a manner unrelated to DNA adduct formation.[34] PAHs are formed during combustion of organic matter such as coal, mineral oil, and oil shale. Therefore, PAH exposure occurs in the form of automobile exhaust, soot, coal tar, cigarette smoke, and charred food products. Many PAHs have been found to be carcinogenic in animal studies, and PAH exposure is associated in humans with lung, skin, and urinary cancers, among others. The carcinogenic potential of PAHs is highly variable. Examples of potent to moderately carcinogenic PAHs include 3-methylcholanthrene, B[*a*]P, dibenzo[*a*,*h*]anthracene, 5-methylchrysene, and dibenz[*a*,*j*]anthracene, whereas benzo[*e*]pyrene, dibenz[*a*,*c*]anthracene, chrysene, benzo[*c*]phenanthrene and fluoranthene are relatively weak or inactive carcinogens. Because humans are exposed to mixtures of PAH that are produced during combustion, estimates of carcinogenic potential associated with diverse exposure scenarios are highly variable.

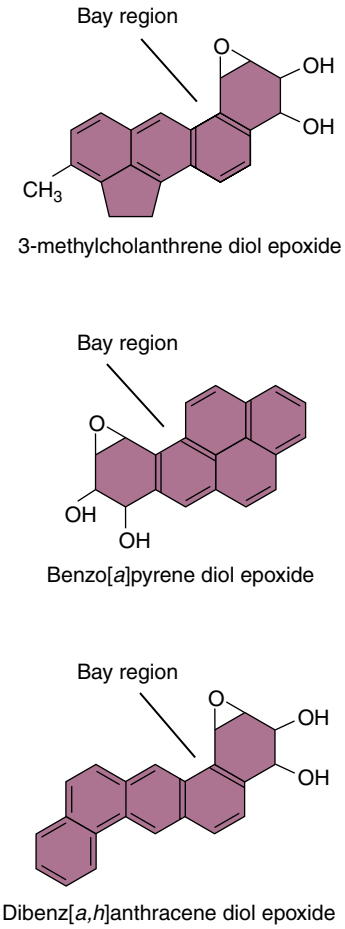


Figure 7-2 Selected polycyclic aromatic hydrocarbon (PAH) bay region dihydrodiol epoxides.

Aflatoxin B_1

One of the most potent liver carcinogens is the fungal metabolite aflatoxin B_1 (AFB_1). AFB_1 and other aflatoxins are produced by *Aspergillus* mold species, such as *Aspergillus flavus* and *Aspergillus parasiticus*. Exposure to aflatoxins occurs via consumption of contaminated nuts and grain, such as peanuts and corn, on which *Aspergillus* species grow. Humid conditions and poor storage contribute to the growth of these molds. In numerous epidemiologic studies, the incidence of hepatocellular carcinoma (HCC) has been correlated with aflatoxin intake. AFB_1 is highly mutagenic in in vitro assays. AFB_1 is converted to an epoxide metabolite responsible for its mutagenic and carcinogenic action. The DNA base targeted by activated AFB_1-epoxide is G (N7 position; see Figure 7-3), and the mutations induced are predominantly GC→TA transversions. Significantly, the *TP53* gene is mutated (GC→TA point mutation in codon 249) in a high proportion of human HCCs that arise in areas where aflatoxin exposure is high.[35,36] Evidence suggests that *TP53* mutation at codon 249 may occur as a result of combined exposure to HBV and AFB_1, and studies have shown elevated risk of HCC in individuals exposed

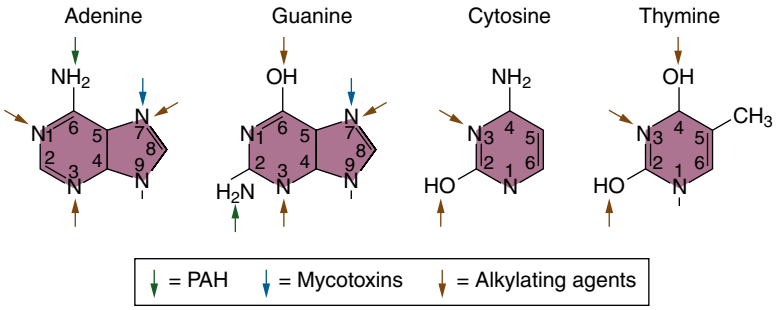


Figure 7-3 Sites of adduct formation associated with carcinogenicity of selected agents.

to both HBV and aflatoxin over individuals exposed to either agent alone.

Benzidine

Benzidine is a member of a large class of carcinogens referred to as *aromatic amines*. The carcinogenic nature of benzidine was discovered in the context of bladder cancer induction in workers in the dye industry.[37] In the past, benzidine-based azo dyes were synthesized in vast quantities in the United States and abroad. In the 1970s, their use was significantly curtailed because of health concerns. However, numerous workers were exposed to these carcinogens before regulation. On activation, benzidine and certain benzidine-based dyes can covalently react with DNA, and benzidine has been shown to induce chromosomal damage in vivo.[38] Benzidine is a bladder carcinogen in multiple species, including humans, dogs, mice, rats, and hamsters, although species differences in activation of the parent compound have made the study of benzidine-induced bladder cancer challenging.[39]

Nitrosamines and Heterocyclic Amines

Shortly after the identification of benzo[*a*]pyrene, *N*-nitrosodimethylamine was shown to induce liver tumors in rats. These results were provocative at the time because of the stark differences in physical properties between the PAHs and the water-soluble *N*-nitroso compounds. Since the initial discovery of *N*-nitrosodimethylamine, a wide variety of *N*-nitroso compounds have been shown to be powerful carcinogens in multiple experimental models and suspected carcinogens in lung and gastrointestinal cancers in humans.[40] Following metabolic activation, *N*-nitrosamines can react with DNA to initiate carcinogenesis. Exogenous and endogenous sources of *N*-nitroso compounds have been described. *N*-nitrosamines are present in smoked meats and in meats containing the antimicrobial and color-enhancing agent nitrite. In both cases, nitrogen oxides are formed, which react with the amines present in meat. Alternatively, the formation of *N*-nitroso compounds can occur endogenously because of low pH conditions in the gastric system or as result of the presence of intestinal bacteria that catalyze *N*-nitroso compound formation.

Heterocyclic amines are also formed in muscle meats on high-temperature processing. Most heterocyclic amines tested are mutagenic in in vitro assays, and several induce gastrointestinal tumors in rodents.[41,42] The two heterocyclic amines found most abundantly in cooked meat and best absorbed into the circulation are 2-amino-1-methyl-6-phenylimidazo-(4,5-*b*)-pyridine (PhIP) and 2-amino-3,8-dimethylimidazo-(4,5-*f*)-quinoxaline (MeIQx). At high temperatures, these heterocyclic amines are formed via reactions among creatinine, creatine, sugars, and amino acids.

N-Nitrosamine exposure is also associated with tobacco use[43]: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and *N*-nitrosonornicotine (NNN) are carcinogenic tobacco-alkaloid–derived *N*-nitrosamines present in unburned and burned tobacco products. PAHs and NNK are the most abundant pulmonary carcinogens in tobacco smoke. In contrast to PAHs, which induce SCCs, NNK induces adenocarcinoma of the lung in animal models. Furthermore, adenocarcinoma of the lung has become the most common lung cancer type in the United States. This fact may reflect changes in cigarette manufacturing in the past 30 to 40 years that have resulted in rising levels of NNK and falling levels of B[*a*]P. In addition, in smokeless tobacco products such as snuff, *N*-nitrosamines are prominent agents involved in the induction of oral cancer. These *N*-nitrosamines require metabolic activation for carcinogenic activity and form DNA adducts similar to other organic carcinogens discussed earlier.

Inorganic Carcinogens

Beryllium

In 1946, Hardy and Tabershaw reported "delayed chemical pneumonias" in workers exposed to beryllium. In that same year, Gardner and Heslington reported experimentally induced osteosarcomas in beryllium-injected rabbits. Subsequent studies in the 1950s demonstrated that inhalation exposure of rodents resulted in induction of lung tumors. Since that time, beryllium has been recognized as a human carcinogen capable of inducing lung cancer in exposed workers. Occupational exposures to beryllium include inhalation of beryllium-containing dusts during processing of ores,

machining of beryllium metal and alloys, and manufacturing of aerospace materials, ceramics, sports equipment, and electronics. Beryllium is weakly mutagenic in bacterial and mammalian mutagenesis test systems; however, it shows strong transformation capacity in Balb/3T3 and Syrian hamster secondary embryo cells.[44] In addition to genotoxic effects, beryllium has been shown to alter the expression of numerous cancer-related genes (i.e., *c-fos*, *c-jun*, *c-ras*, MAP kinases, DNA repair genes,). Despite these reports, the carcinogenicity of beryllium has recently been called into question.[45,46] Current occupational exposure levels are much lower than historical values, and previous reports of beryllium's effects may have been confounded by inadequate smoking history information.

Cadmium

Cadmium is a heavy metal present in soil, air, and water and is listed as a priority pollutant by the U.S. EPA. Occupational exposures to cadmium occur during the manufacture of nickel-cadmium batteries, pigments, and plastic stabilizers as well as electroplating processes, metal smelting, and electronic waste recycling.[47] In addition, cigarette smoke contains cadmium. Release of industrial cadmium waste into the environment is of particular concern because of its long biologic half-life. On absorption, cadmium can accumulate in the body because it is poorly excreted and effectively stored in liver and kidney as a result of binding to metallothionein. Furthermore, once absorbed, no effective detoxification pathways for cadmium exist. The half-life of cadmium in humans is estimated at 15 to 20 years.

Cadmium exposure has been linked to human lung cancer and may affect the risk of prostate and kidney cancers. Although the carcinogenicity of cadmium has been confirmed in rodent models, the precise mechanism is unknown.[47] Cadmium binds only weakly to DNA and is only weakly mutagenic in bacterial and mammalian assays, and high concentrations are required to induce oxidative stress. Cadmium may act via non-genotoxic mechanisms to activate proto-oncogenes and disrupt normal cellular processes. For example, cadmium has been shown to alter E-cadherin–mediated cell adhesion, inhibit DNA repair, and alter expression of numerous genes in vitro including *c-fos*, *c-myc*, metallothionein, and genes encoding heat shock proteins.

Arsenic

Arsenic is widely distributed in the environment, being found in the earth's crust in both inorganic [arsenite-As(III) and arsenate-As(V)] and methylated forms [monomethylated arsenic (MMA) and dimethylated arsenic (DMA)]. As(III), as well as MMA(III) and DMA(III), have been associated with skin, lung, urinary bladder, kidney, and liver cancers.[48] Human exposure to arsenic occurs via contaminated drinking water, diet, or contact with wood preserved with arsenicals; during mining of tin, gold, and uranium; and during application of arsenical pesticides. Signs of chronic exposure to arsenic in drinking water include altered skin pigmentation and hyperkeratosis of the palms of the hand and soles of the feet, which may ultimately lead to skin lesions and skin cancer.

Much attention has been given to assessing the health impact of arsenic contamination in drinking water sources. The current WHO guidelines for arsenic exposure recommend no more than 10 μg/L arsenic in water intended for human consumption. Since the 1980s, millions of people in China, India, Bangladesh, the United States, Chile, and Argentina have been exposed to arsenic in the drinking water far in excess of this limit. Already, numerous epidemiologic studies in Taiwan, the United States, Chile, and Argentina have demonstrated excess cancer risk in areas with known high exposure to arsenic in drinking water.[49] Unfortunately, identifying a safe level of arsenic in drinking water has been difficult because most epidemiologic studies show adverse effects at high doses; data concerning health risk at low exposures are unavailable. After intense debate, the limit in the United States was lowered to 10 μg/L in 2006.

For years, the study of arsenic and cancer was hindered by a lack of experimental evidence of carcinogenicity in animals. Only recently have studies of methylated arsenic and early life exposures provided adequate validation in vivo. As(III) and As(V) are transported into cells, As(III) more readily than As(V). On absorption, As(V) is reduced to As(III); As(III) can then be methylated. Historically, methylation of As(III) was considered to be a detoxification reaction, but recent evidence contradicts this dogma.[50] MMA(III) and DMA(III) are at least as cytotoxic, mutagenic, and clastogenic as As(III). Nonetheless, when methylated, arsenic is readily excreted in urine. DMA can be detected in urine shortly after exposure; also, because of the wide distribution of arsenic, exposure can be assessed via hair and fingernail deposits months or years after exposure. Therefore, methylated arsenic in hair and fingernail samples serves as a useful biomarker of exposure.

Numerous mechanisms of action have been proposed for arsenic carcinogenicity.[48] Arsenic exposure is known to generate reactive oxygen species. Like many transition metals, arsenic can participate in Fenton reactions that produce oxidative stress. Furthermore, arsenic may activate superoxide-generating NAD(P)H oxidase. In this way, arsenic is thought to induce DNA and protein damage that may initiate carcinogenesis. Arsenic has also been shown to elevate the total level of tyrosine phosphorylation in cells. Specifically, arsenic may alter phosphorylation-dependent epidermal growth factor receptor (EGFR) and mitogen-activated

protein kinase (MAPK) signaling. In addition, arsenic has been shown to alter NFκ signaling, apoptosis rates, cell cycle regulation, DNA repair, and genome stability. More recent evidence suggests that epigenetic dysregulation may underlie these adverse effects of arsenic exposure.[51]

Chromium

Chromium in the hexavalent state [Cr(VI)] is a human carcinogen. The carcinogenic properties of chromium have been identified via epidemiologic studies of exposed workers in industries such as chrome plating, welding, leather tanning, and stainless steel production. Exposure to chromium generally occurs via inhalation and primarily affects risk of lung cancer. Because of environmental contamination, consumption of chromium in drinking water is also possible; however, the health consequences of the low-level exposure are unclear.[52]

The oxidation state of chromium determines not only its bioavailability but also its cellular reactivity.[53] Cr(VI) readily enters cells via anion channels, whereas Cr(III) only slowly crosses the cell membrane. On entry to the cell, Cr(VI) is likely reduced, as Cr(VI) does not readily react with DNA in in vitro analyses. Chromium in lower oxidation states [Cr(III), Cr(IV), and Cr(V)] is more reactive; Cr(III) is believed to be the ultimate DNA reactive form. The reduced forms of chromium can also induce oxidative stress. In addition to or as a result of oxidative stress, chromium alters cell signaling pathways. Signaling molecules affected include NFκB, AP-1, p53, and HIF-1.

Fibers

Asbestos

The term *asbestos* refers to a group of naturally occurring silicate mineral fibers. There are numerous types of asbestos fibers that are classified according to their morphologic characteristics, including whether the fibers are curly (serpentine) or straight (amphibole). The shape and length-to-width ratio are important determinants of whether a particular asbestos fiber type will be carcinogenic.[54] This is likely because the size of the fiber determines the ability of the fiber to reach the deep lung tissues and penetrate the lung. Long (>4 μm) and thin (<0.5 μm diameter) fibers are the most carcinogenic. Extensive exposure to asbestos has occurred because the flame-resistant and durable characteristics of asbestos have led to its use as an insulating agent in schools, factories, homes, and ships, as construction material, and as a raw material for automobile brake and clutch parts. A large cohort of workers was exposed to high levels of asbestos when ship building peaked during World War II.

The toxic effects of asbestos exposure have been known for many years.[54] For example, more than 40 years ago, crocidolite asbestos exposure of South African miners was linked to mesothelioma incidence. Mesothelioma is a rare cancer of the membranous lining of the abdomen and chest. Numerous animal studies and in vitro experiments support the conclusion that asbestos can induce tumors. In fact, few cancer cause-effect relationships are as striking as asbestos and mesothelioma; most cases of mesothelioma can be related to asbestos exposure. In addition to mesothelioma, asbestos exposure has been associated with lung and laryngeal cancers. The initial accumulation of evidence of asbestos carcinogenicity was obscured by the differences in carcinogenicity of asbestos fibers of varying shape and by the long latency for the development of tumors following exposure. Since identification of asbestos as a cancer-causing agent, asbestos usage in the United States has greatly declined because of the introduction of a replacement material (fiberglass) and OSHA regulation of asbestos exposure. Nonetheless, mesothelioma rates have not declined in the United States in the past 15 years.

Numerous biologic hypotheses concerning the mechanism(s) by which asbestos induces tumors have been proposed.[55] Because long, thin fibers are the most carcinogenic, asbestos fibers may penetrate the lung and irritate the lining of the chest wall. The chronic inflammation and scarring would then contribute to tumor formation. Alternatively, the fibers may pierce spindle fibers during mitosis and thereby induce chromosome damage. Finally, asbestos fibers may induce oxidative stress and/or alter EGFR and MAPK cell signaling. Significantly, epidemiologic studies show that cigarette smoking acts synergistically with asbestos exposure to induce lung tumors. In addition to asbestos, exposure to other fibers such as plant-derived silica fibers (biogenic silica) has been shown to be carcinogenic.[56]

Hormones

The etiology of numerous cancers is believed to be influenced by hormonal or dietary factors, and hormones under certain conditions are considered to be known human carcinogens (see Table 7-5). As previously mentioned, overweight and obesity are associated with elevated cancer risk. This effect may be mediated by endocrine dysregulation such as altered adiponectin, leptin, insulin, and IGF-1 levels. In addition, prostate, ovarian, breast, testicular, and endometrial cancers are hormonally driven.[57,58] A role for hormones in cancer etiology was established when castration and ovariectomy studies revealed that hormone-dependent cancers could be prevented by removing the primary hormone-synthesis organs. As an example of the action of hormones in cancer formation, estrogen activates hormone-responsive receptors. Stimulation of these receptors, such as the estrogen receptors, can increase the cellular proliferation rate to promote tumorigenesis. Endogenously synthesized hormones and administered hormones have been shown to influence cancer formation. Hormone replacement therapy and estrogen-only birth control therapy have been associated

with increased risk of hormone-dependent cancers. An even more dramatic example of synthetic hormone-induced cancer is that of women who were exposed to estrogenic diethylstilbestrol (DES) in utero. DES was taken by pregnant women to prevent abortion; however, a large percentage of their female offspring developed clear-cell carcinomas of the vagina and cervix after the onset of puberty.

Mechanisms of Chemical Carcinogenesis

Multistage Nature of Carcinogenesis and the Multistage Model of Mouse Skin Carcinogenesis

In the early days of carcinogenesis research, it was noted that wounding of the skin of mice previously treated with mutagenic coal tar led to skin tumor formation. To explain these findings, a multistage model of carcinogenesis was proposed.[59] The model holds that tumors arise in cells that have first undergone a mutating event initiated by an electrophilic metabolite such as that formed from benzo[*a*]pyrene found in coal tar. Subsequently, cell proliferative stimuli promote the initiated cell population to expand, resulting in premalignant clonal outgrowths. Finally, additional genetic alterations accumulate in these lesions, leading to the development of a neoplasm that becomes invasive and ultimately metastatic. Over the years, this model has been refined to encompass the fundamental role of stem cells as the targets of initiation, the importance of stemlike characteristics in the plasticity of developing tumors, and the critical role of tumor microenvironment in carcinogenesis.[60] In addition, the mechanistic importance of DNA methylation changes and histone modifications in the initiation, promotion, and progression phases has been acknowledged.

Numerous animal models have been developed to study the multistep manner in which various epithelial and other tumors develop and progress. In one of the best-characterized models, the mouse two-stage skin carcinogenesis model, a subcarcinogenic dose of a mutating agent is delivered.[61] This is followed by multiple exposures to growth-promoting stimuli and the appearance of tumors on the dorsal skin (Figure 7-4). This model has provided an excellent paradigm in which to examine the carcinogenic potential of environmental agents and has been used to reveal the mechanistic bases of multistage carcinogenesis by environmental agents.

Initiation and Mutational Theory of Carcinogenesis

During the first stage of multistage carcinogenesis, DNA mutations result as a consequence of electrophilic carcinogen exposure, oxidative damage to DNA, DNA strand breaks, or other DNA insults. Mutations are believed to occur in multipotent stem cells and are inherited by daughter cells. Theodor Boveri first proposed the concept that cancer arises as a result of damage to genetic material at the turn of the 20th century. In the 1950s and 1960s, James and Elizabeth Miller, after observing that a wide variety of structurally diverse chemicals could induce cancer in animal models, suggested that chemical carcinogens required metabolic activation to electrophilic intermediates. These electrophilic intermediates could then covalently bind to proteins, RNA and DNA.

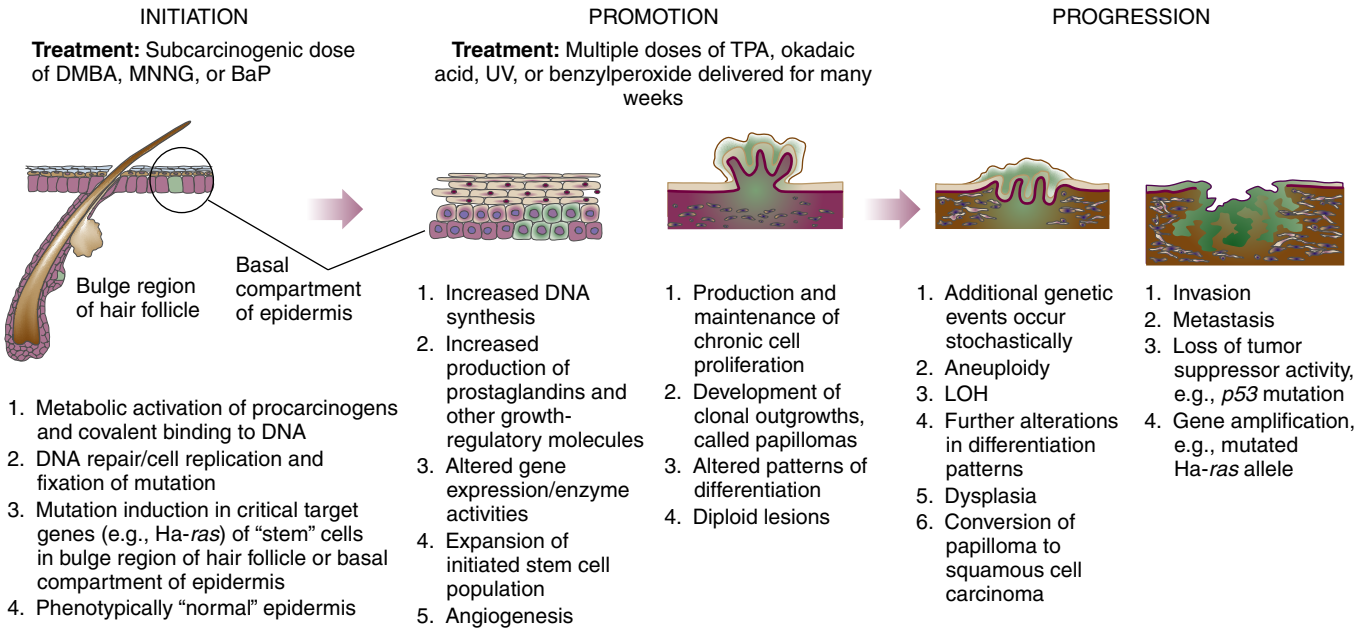


Figure 7-4 Multistage model of mouse skin carcinogenesis. *BaP,* Benzo[*a*]pyrene; *DMBA,* 7,12-dimethylbenz[*a*]anthracene; *LOH,* loss of heterozygosity; *MNNG, N*-methyl-*N*-nitro-*N*-nitrosoguanidine; *TPA,* 12-*O*-tetradecanoyl phorbol-13-acetate.

The term *electrophile theory of chemical carcinogenesis* was coined to describe their concept. The Millers' work was supported by data reported in 1964 by Brooks and Lawly, which demonstrated that the degree of covalent binding of carcinogenic PAHs to DNA correlated with carcinogenic potential.[62] Subsequently, many chemical carcinogens have been shown to bind and alter DNA integrity, thereby inducing mutations. Carcinogens that alter DNA to induce cancer in this manner are referred to as *genotoxic carcinogens*. The majority of carcinogens identified to date are genotoxic carcinogens.

DNA mutations occurring in proto-oncogenes and tumor suppressor genes are particularly critical to the initiation of carcinogenesis. These normal cellular genes are targeted during carcinogenesis and play critical roles in tumor formation. Proto-oncogene mutations are dominant, in that activation of a single copy of a proto-oncogene to an oncogene may be significant for carcinogenesis. Proto-oncogenes are discussed in greater detail in Chapter 2; however, a list of proto-oncogenes and the cancers with which they are associated is provided in Table 7-6. In contrast to proto-oncogenes, the normal cellular function of tumor suppressor genes is to negatively regulate cell growth. According to Knudson's two-hit theory, tumor suppressor genes require that both copies of the gene be lost or inactivated because tumor suppressor mutations are recessive in nature. For instance, inheritance of one mutated copy of *p53* is not significant until the second copy is lost ("second hit"), resulting in loss of heterozygosity (LOH). Examples of tumor suppressor genes and associated cancers are also provided in Table 7-6.

In the multistage mouse skin carcinogenesis model, initiation occurs via application of a genotoxic carcinogen (e.g., *N*-methyl-*N*-nitro-*N*-nitrosoguanidine [MNNG], 7,12-dimethylbenz[*a*]anthracene [DMBA], or B[*a*]P; see Figure 7-4). A subcarcinogenic dose of the initiating agent is applied to the shaven dorsal skin of the mouse. The critical mutations for tumor development are believed to occur in epidermal multipotent stem cells, a major population of which resides in the bulge region of the hair follicle. Although no phenotypically aberrant cells are apparent in the "initiated" skin, small populations of epidermal cells can be identified as early as 1 week after treatment with DMBA that contain signature mutations. The initiation stage is irreversible and cumulative. That is, the dose required for initiation can be divided and applied in portions over time or applied in a single dose with essentially the same result. In addition, commencement of the promotion phase can be delayed, because the DNA mutations induced by the initiating agent are permanent.

Table 7-6 Selected Proto-oncogenes and Tumor Suppressor Genes and Some Associated Cancers

Gene Type	Gene Name	Associated Cancer Type
Proto-oncogene	*C-MYC*	Leukemia, lung, colon
	ERBB-2	Breast, ovary
	ABL	Leukemia
	RASH	Bladder
	RASK	Lung, ovary, bladder
	RASN	Breast
Tumor suppressor	*APC*	Colon
	P53	Breast, colon, lung
	RB	Retinoblastoma, breast, bladder, lung
	$BRCA_1$	Breast, ovary
	$BRCA_2$	Breast

Adapted from Perkins AS, Stern DF: Molecular biology of cancer. Oncogenes. In: DeVita VT, Hellman S, Rosenbert SA, eds. *Cancer: Principles & Practice of Oncology*. 5th ed. Philadelphia, Pa: Lippincott-Raven Publishers; 1997.

In the mouse skin model, the most frequently mutated proto-oncogene following initiation with PAH is *Hras1* (reviewed in Ref. 61). Mutations at G^{38} of codon 13, in the case of B[*a*]P, and at A^{182} of codon 61, in the case of DMBA, lead to constitutive activation of the gene product of *Hras1*. These mutations can be detected in the resulting tumors, reflecting the clonal origin of the papillomas. In addition, the specificity of these mutations is directly related to the major sites of DNA adduct formation arising from the carcinogenic diol-epoxides of these two PAH carcinogens. In a variety of rodent models of multistage cancer (rat azoxymethane-induced colonic lesions, mouse diethylnitrosamine-induced liver foci, mouse urethane-induced lung adenomas), mutations in ras oncogenes frequently occur, highlighting the importance of ras, and oncogenes in general, in the development of cancer. Activating mutations in *Hras1* are believed to confer, at least in part, resistance to terminal differentiation of keratinocytes during tumor promoter treatment, thus conferring a selective growth advantage to these cells. Findings in animal models have established the irreversible and cumulative nature of tumor initiation and underscore the specificity of critical DNA mutations in proto-oncogenes or tumor suppressors induced by genotoxic carcinogens.

During carcinogenesis, numerous types of DNA lesions occur following exposure to carcinogenic agents. For example, in the case of electrophilic carcinogen attack, specific points within the DNA nucleotides are targeted for adduction (see Figure 7-3). As noted previously, B[*a*]P targets primarily the N^2 exocyclic amino group of guanine, whereas other PAHs may target adenine in addition to guanine (e.g., DMBA). As shown in Figure 7-3, alkylating agents target numerous sites within DNA bases. However, certain sites (e.g., 0^6 methylguanine and 0^4 methylthymine for methylating agents) may be the most important for carcinogenesis by this class of carcinogen. During replication, mispairing due to

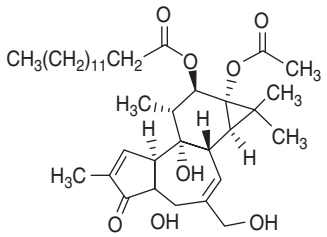
DNA adducts or other DNA lesions may become fixed, and the ultimate effect depends on the location of the mutation. Mutations affect coding sequences, intronic signals, untranslated regions, or promoter elements; consequently, protein function or expression levels may be altered. Following DNA double-strand breaks, incorrect rejoining of DNA has been shown to cause rearrangement of DNA coding and promoter regions. In addition to these qualitative changes, quantitative changes in gene copy number (gene amplification or gene deletion) may also affect key cancer-associated genes.

Promotion

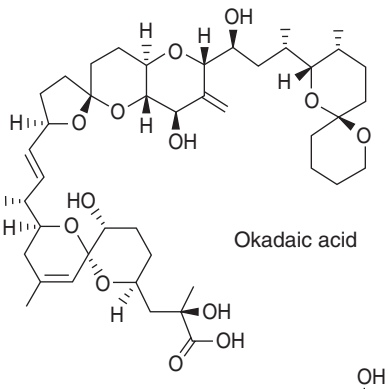
During the tumor promotion phase of carcinogenesis, growth stimuli and other factors promote clonal expansion of initiated cells. This stage is characterized by altered gene expression and proliferation of initiated cells, some of which maintain stemlike characteristics. Most tumor promoters are thought to exert their effects through cellular receptors or cell growth, differentiation, and/or apoptotic signaling pathways. Inflammatory mediators or other stromal factors may mediate these effects by providing a permissive environment for tumor growth. Promoting agents do not directly affect DNA but act primarily via non-genotoxic, reversible mechanisms.

Growth Factor Receptor Signaling Pathway Engagement

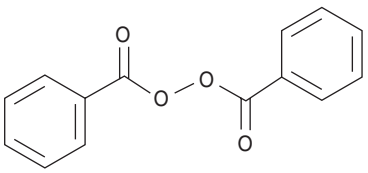
In the mouse two-stage skin carcinogenesis model, the promotion stage is elicited by multiple applications of promoting agents delivered over the course of weeks or months (see Figure 7-4). In this model, promotion must occur following initiation with a mutating agent. As opposed to initiation, the promotion stage is initially reversible, does not elicit DNA mutation, is prolonged in nature, and appears to be nonadditive. Typical skin tumor-promoting agents include the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA); the phosphatase inhibitor okadaic acid; the organic peroxide benzoyl peroxide; and the anthrone derivative chrysarobin (see Figure 7-5 for the diversity of structures of chemical tumor promoters). In addition, UV light, repeated



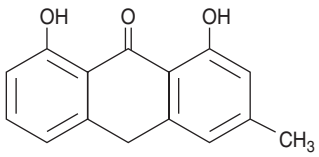
Phorbol 12-myristate 13-acetate (TPA)



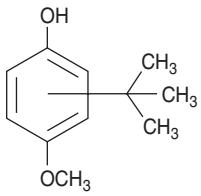
Okadaic acid



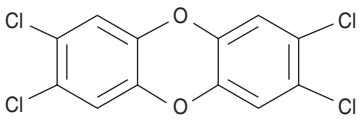
Benzoyl peroxide (BzP)



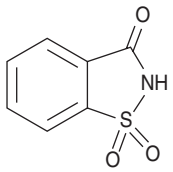
1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin)



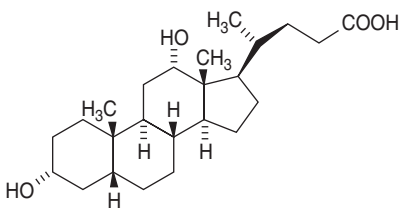
Butylated hydroxyanisole (BHA)



2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)



Saccharin



Deoxycholic acid

Figure 7-5 Chemical structures of selected tumor promoters.

abrasion, full-thickness wounding, and certain silica fibers possess the ability to function as tumor promoters. The endpoint of promotion in the mouse two-stage skin carcinogenesis model is the development of premalignant, clonal outgrowths referred to as *squamous papillomas*. These lesions (hyperplastic epidermis folded over a core of stroma) are still well differentiated and do not possess the ability to invade surrounding tissue. Once the cells of the papilloma acquire additional mutations that allow autonomous growth, the promotion stage is no longer reversible.

Promoting agents act primarily by altering gene expression.[63] The initial interaction of promoting agent with the cell depends on the nature of the promoter. In the mouse skin model, the receptor for TPA (the most frequently used promoting agent) has been identified as protein kinase C (PKC). Stimulation of PKC results in a cascade of events that allow for expansion of the initiated cell population. PKC-mediated signaling events include induction of ornithine decarboxylase (ODC) activity, activation of the MAPK pathway, and upregulation of ligands for the EGFR. EGFR activation leads to further activation of multiple signaling pathways involved in proliferation and survival. For instance, Akt and Stat3 signaling are believed to affect cell-cycle parameters via altered cyclin Dl expression.[64,65] Although the initial mechanism for other skin tumor promoters (e.g., okadaic acid, benzoyl peroxide, chrysarobin) is different from that of TPA, all tumors promoter ultimately elicit key biologic and molecular changes. These changes include induction of ODC, induction of growth factors and cytokines, production of eicosanoids, and increased DNA synthesis. Growth factors and cytokines known to be altered by tumor-promoting stimuli include TGF-α, TGF-β, IL-1, IL-6, and TNF-α, among others.

During tumor promotion, immune inflammatory cells play diverse and complex roles. Increasingly, cytokines are acknowledged as active players in the growth-promoting environment of developing neoplasia. These cells, along with fibroblasts and other cells present in the stroma, release signaling molecules such as EGF, VEGF, and FGF2 to facilitate proliferation and contribute to proinvasive capacity by affecting expression of matrix metalloproteinases and other proteases. In contrast, other immune surveillance cells can play tumor-antagonizing roles.

Examples of Tumor Promoters

Tumor-promoting agents have been identified for a number of rodent tissues other than mouse skin, indicating the generality of this phenomenon to other organs and species (reviewed in Ref. 66). Some examples of tumor promoters that act on organs other than skin include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (liver), butylated hydroxyanisole (BHA) (lung), sodium saccharin (urinary bladder), and bile acids (colon) (see Figure 7-5 again for chemical structures). Certain components of tobacco smoke can also act as tumor promoters.

Progression

The final stage of carcinogenesis is referred to as *progression*. The original tumor mass increases in size, additional mutations accumulate, and invasion and metastasis occur. Genome stability is compromised, and a mutator phenotype may develop to permit further accumulation of mutations. Crucial to progression of solid tumors is the ability of cancer cells to invade the surrounding stroma, enter the bloodstream, and extravasate to colonize distal sites. For cells to break away from the primary tumor, downregulation of cell adhesion, often by repression of E-cadherin expression, must occur. Subsequently, the cells must acquire mobility and ability to invade the surrounding stroma and blood vessel basement membranes. Invasion requires the action of degradation enzymes such as the matrix metalloproteinases (MMPs). These changes are collectively referred to as the *epithelial-to-mesenchymal transition* (EMT). Once established, the metastatic colony must develop adequate nutrition and oxygen supply via stimulation of angiogenesis.

The process whereby normal cells accrue an increasingly aberrant and invasive phenotype as a consequence of selection has been likened to evolutionary theory at the population level.[67] However, in addition to an accumulation of genetic mutations, tumor promotion and tumor progression can also involve heritable but non-genotoxic lesions. These changes, referred to as *epigenetic reprogramming*, involve altered DNA methylation patterns and changes to the histone code. In the case of DNA methylation, the promoter regions of tumor suppressor genes may become hypermethylated, thereby silencing gene expression. Alternatively, the expression of proto-oncogenes may be upregulated via hypomethylation. Recent proposals suggest that environmental agents elicit epigenetic changes and alter the risk of tumor promotion and/or progression.[68,69] Interestingly, epigenetic reprogramming may precede the neoplastic process in some cases, and modifications to the histone code may affect DNA damage response or DNA repair during initiation, promotion, or progression.[69]

During progression in the two-stage mouse skin carcinogenesis model, premalignant papillomas convert to SCCs (see Figure 7-4).[61] This conversion process is accompanied by additional genetic alterations, including the development of aneuploidy. Characteristic changes in gene expression such as elevation in gamma-glutamyltranspeptidase, $\alpha_6\beta_4$ integrin, and keratin-13 expression as well as loss of E-cadherin expression are also common. The histologic appearance of SCCs can be distinguished from papillomas by downward growth and loss of ordered differentiation of epidermal keratinocytes.

Multistage Environmental Carcinogenesis in Humans

The applicability of multistage carcinogenesis concepts to human cancer is supported by a number of observations. First, human environmental carcinogen exposure outside of occupational settings usually occurs in low doses repeatedly delivered over the course of months or years. Each individual dose alone is likely insufficient to produce cancer. In addition, it is unlikely that a single dose of a sole agent is the cause of most human cancers. Second, there is considerable evidence from human epidemiologic and experimental animal studies that certain human carcinogens such as tobacco smoke and UV light exhibit a strong tumor-promoting activity. Furthermore, many components of the human diet appear to influence cancer in humans through a tumor-promotion type of effect. Finally, histochemical and molecular examination of tumors at various stages indicates that human cancers develop via multiple steps. It has been postulated that human cancers require as many as four to six sequential genetic events for their development.[70]

Numerous human cancers, particularly those of epithelial origin, appear to develop in a multistage progression. For instance, regions of dysplasia and carcinoma in situ appear to precede invasive carcinoma when melanoma, head and neck squamous-cell carcinoma, and cervical cancer lesions are examined. Supporting the multistage nature of cancer development, genetic alterations have been shown to accumulate during tumorigenesis in these lesions. For example, during colorectal carcinogenesis, mutations in the adenomatous polyposis coli (APC) gene appear to initiate tumorigenesis.[71] A portion of the resulting dysplastic foci further accumulate mutations in the K-*ras* oncogene and other oncogene and tumor suppressor genes and progress from adenomas to invasive carcinomas. A similar pattern of accumulation of molecular abnormalities has been noted for squamous-cell lung carcinoma. As the severity of the histopathologic appearance of these lesions increases, the frequency of loss of heterozygosity events also increases.[72]

Examination of tumor DNA has also validated a role for environmental carcinogens in the etiology of human cancer. When tumor suppressor and oncogene gene sequences are examined, characteristic mutation spectrums can be identified and associated with specific carcinogen exposure. The mutation spectrum of the *p53* tumor suppressor gene has been intensively studied.[73] A database of more than 10,000 reports of *p53* mutations in human cancers has been collected. Depending on the cancer type, mutations are frequently reported at amino acids 130-142, 151-164, 171-181, 193-200, 213-223, 234-258, and 270-286, which are part of the DNA-binding domain of *p53*. Sixty-one percent of lung cancer samples have mutations at codon 157 in addition to mutations in codons 248 and 273. A large percentage of these mutations are the result of G→T transversions. In vitro analyses indicate that exposure of normal human bronchiolar epithelial cells to benzo[*a*]pyrene diol epoxide results in DNA adducts in *p53* in the same mutation hot spots as in lung cancer: codons 157, 248, and 273.[74] These results provide strong evidence for a link between chemical carcinogen exposure (B[*a*]P of cigarette smoke) and human lung cancer. In addition, aflatoxin B_1 exposure correlates strongly with liver cancer and *p53* mutation at codon 249, whereas sunlight exposure, which is known to induce CC→TT transition mutations, correlates with CC→TT tandem mutations at hot spots for skin cancer in *p53*.

Endogenous Defense Systems against Chemical Carcinogenesis

Various genetically influenced defense systems determine the ultimate outcome following exposure to an environmental carcinogen. Chemical carcinogens are often metabolized to less toxic derivatives and cleared from the body. Damage to cellular DNA may be repaired, or the damaged cell may be eliminated via apoptosis. Furthermore, the cell possesses a number of endogenous defense mechanisms against carcinogen-induced oxidative stress. Interindividual differences in the efficacy of these defense mechanisms are known to exist and influence host susceptibility to environmentally induced cancer.

Carcinogen Metabolism

Subsequent to absorption through the gastrointestinal tract, xenobiotics travel via the portal vein to the liver where first-pass metabolism occurs. Hepatic tissues are highly concentrated with metabolic enzymes specialized in chemical conversion referred to as *biotransformation*. In other cases, such as inhalation or dermal exposure, biotransformation enzymes at the site of exposure can begin immediately to convert the parent compound into metabolites. Biotransformation enzymes are theorized to have evolved primarily as natural defenses against environmental chemical exposure.

Phase I and Phase II Biotransformation Reactions

The reactions catalyzed by biotransformation enzymes have been categorized into groups referred to as *phase I* and *phase II* reactions because of their often sequential roles in the conversion of xenobiotics. Phase I reactions include oxidation, reduction, and hydrolysis reactions and, generally, expose functional groups that enable phase II biotransformation to proceed. Phase II biotransformation reactions catalyze

glucuronidation, sulfation, acetylation, methylation, and glutathione conjugation reactions, among others.[75] Numerous enzymes that catalyze these reactions have been identified and classified according to gene family (Table 7-7).

Phase I metabolites, in general, display minimally increased hydrophilicity. In contrast, phase II biotransformation reactions catalyze the addition of cofactor molecules to the parent compound, resulting in a significant increase in hydrophilicity. In certain instances, phase II conjugation reactions may also target the parent compound for export via specialized efflux pumps. Therefore, in general, phase II biotransformation reactions ultimately result in metabolites that are less toxic and more readily excreted. In contrast, phase I biotransformation of carcinogens often results in reactive metabolites capable of covalent modification of cellular macromolecules. It is important to note, however, that these are generalizations. Examples of phase I–mediated detoxification have been noted, and phase II–mediated chemical activation has been documented.

According to the Millers' electrophilic theory of carcinogenesis, all mutagenic compounds must be inherently chemically reactive or converted via biotransformation to a reactive form. Carcinogens that do not require metabolic activation are referred to as *direct carcinogens;* indirect carcinogens require metabolic activation. The conversion of the parent compound to a reactive state converts a procarcinogen to an ultimate carcinogen. Ultimate carcinogens, like direct carcinogens, are electrophilic and attack nucleophilic groups in DNA to initiate carcinogenesis, as discussed in the section titled Initiation and Mutational Theory of Carcinogenesis. Although categorizing biotransformation reactions according to the phase I versus phase II nature of the metabolism is useful, the endpoint of carcinogen exposure is often determined by a combination of oxidation, reduction, and conjugation reactions.

PAH Biotransformation

PAHs are widely studied substrates for cytochrome P450 (CYP450)-mediated biotransformation. CYP450s, a class of enzymes present in the endoplasmic reticulum of most cells, have been implicated in numerous carcinogen activation reactions. In humans, the CYP450 family consists of more than 50 genes. which are grouped on the basis of sequence similarity into families (1, 2, 3, . . .), subfamilies (A, B, C, . . .), and individual CYP450s (1, 2, 3, . . .) (e.g., CYP450 1A1, 1A2, 1B1, etc.).[64] CYP450s catalyze oxidation, reduction, oxygenation, dealkylation, desulfuration, dehalogenation, and hydroxylation reactions. CYP450-mediated reactions can detoxify direct carcinogens or activate indirect carcinogens.

Once absorbed, certain PAHs can be biotransformed into electrophilic mutagens via the sequential action of phase I enzymes (Figure 7-6[23]). First, PAH double-bond oxidation is catalyzed by CYP450 enzymes (e.g., CYP1A1). For example, in the case of B[*a*]P, CYP450-mediated oxidation forms the epoxide intermediate, benzo[*a*]pyrene-(7*R*,8*S*)-epoxide. Next, microsomal epoxide hydrolase (mEH) catalyzes hydrolysis of arene oxide to a *trans* dihydrodiol. Finally, a CYP450-catalyzed oxidation reaction forms the ultimate carcinogen (i.e., benzo[*a*]pyrene-7,8 diol-9,10-epoxide), a diol-epoxide metabolite. In human lung tissue, both B[*a*]P epoxidation steps are catalyzed primarily by CYP1A1. Studies using CYP1B1-deficient mice highlight the importance of this p450 enzyme in PAH activation. These mice are resistant to DMBA-induced carcinogenesis, due to a lack of conversion of DMBA from procarcinogen to ultimate carcinogen.[76]

PAHs can be detoxified by glutathione *S*-transferases (GSTs). GST-mediated glutathione conjugation of PAH epoxides can deactivate the ultimate carcinogen, prevent activation to reactive diol epoxides, or accelerate clearance of PAHs following exposure. In this way, GSTs act as an endogenous defense system against PAH-induced carcinogenesis.

Aflatoxin Biotransformation

Metabolism plays a critical role in determining the carcinogenicity of the mycotoxin AFB_1. AFB_1 must first be activated to the ultimate carcinogen, *exo*-8,9-AFB_1-epoxide (see Figure 7-6). This reaction is predominantly catalyzed by CYP450 3A4 in humans.[35] Alternatively, CYP450s can metabolize AFB_1 to inactive products such as AFM_1, AFQ_1, or AFB_1 *endo*-8,9-epoxide (AFBO).

Glutathione conjugation catalyzed by GSTs plays a critical role in protecting against mutagenic and carcinogenic effects of AFB_1 metabolites. Generally, GSTs facilitate xenobiotic clearance by catalyzing glutathione conjugation of a variety of electrophilic substrates.[77] In humans, cytosolic GSTs are categorized according to gene sequence similarity into at least six classes: Alpha (A), Mu (M), Omega (O), Pi (P), Theta (T), and Zeta (Z). Individual GST family members demonstrated unique, though overlapping, substrate specificity.

Subsequent to activation, GST-mediated glutathione (GSH) conjugation can detoxify the AFB_1 epoxide, and this reaction is a major factor underlying the substantial species variation in sensitivity to AFB_1-induced carcinogenesis. For example, rats are highly sensitive to AFB_1-induced hepatocarcinogenesis, whereas mice are comparatively resistant. In line with this observation, mice express mGSTA3-3, which demonstrates high activity toward AFBO, whereas rat GST-mediated deactivation of AFB is significantly less in comparison. Mutational studies of recombinant mGSTA3-3 indicate that the high activity of this protein toward AFBO is due to multiple, critical amino acid residues in the substrate binding site that are not present in homologous rat GSTA3-3.[78]

Table 7-7 Selected Phase I and Phase II Biotransformation Enzymes

Enzyme Classification	Reaction Catalyzed	Gene Family	Class/ Subfamily	Isoforms
Phase I	Oxidation, others	Cytochrome P450	CYP_1	CYP_1A_1
				CYP_1A_2
				CYP_1B_1
			CYP_2	CYP_2A_6
				CYP_2A_{13}
				CYP_2B_6
				CYP_2C_8
				CYP_2C_9
				CYP_2C_{19}
				CYP_2D_6
				CYP_2E_1
			CYP_3	CYP_3A_4
				CYP_3A_5
				CYP_3A_7
				CYP_3A_{43}
			CYP_4	CYP_4A_{11}
				CYP_4A_{22}
				CYP_4B_1
			$CYP_{>4}$	CYP_5AL
				CYP_8AL
				$CYP_{19}AL$
				$CYP_{21}A_2$
	Hydrolysis	Epoxide hydrolase	Microsomal	$EPHX_1$
			Cytosolic	$EPHX_2$
Phase II	Glutathionylation	Glutathione *S*-transferase	Alpha	$GSTA_1$
				$GSTA_2$
				$GSTA_3$
				$GSTA_4$
				$GSTA_5$
			Mu	$GSTM_1$
				$GSTM_2$
				$GSTM_3$
				$GSTM_4$
				$GSTM_5$
			Omega	$GSTO_1$
				$GSTO_2$
			Pi	$GSTP_1$
			Theta	$GSTT_1$
				$GSTT_2$
			Zeta	$GSTZ_1$
	Acetylation	*N*-acetyltransferase		NAT_1
				NAT_2
	Sulfation	Sulfotransferases	$SULT_1$	$SULT_1A_1$
				$SULT_1A_2$
				$SULT_1A_3$
				$SULT_1B_1$
				$SULT_1C_2$
				$SULT_1C_4$
				$SULT_1E_1$
			$SULT_2$	$SULT_2A_1$
				$SULT_2B_1$
			$SULT_4$	$SULT_4A_1$
	Methylation	Catechol-*O*-methyltransferase	Soluble	S-COMT
			Membrane-bound	MB-COMT
	Glucuronidation	UDP-glucuronosyltransferases	UGT_1	UGT_1A_1
				UGT_1A_3
				UGT_1A_4
				UGT_1A_5
				UGT_1A_6
				UGT_1A_7
				UGT_1A_8
				UGT_1A_9
				UGT_1A_{10}
			UGT_2	UGT_2A_1
				UGT_2A_2
				UGT_2B_4
				UGT_2B_7
				UGT_2B_{10}
				UGT_2B_{15}
				UGT_2B_{17}
				UGT_2B_{28}
			UGT_3	UGT_3A_1
				UGT_3A_2
			UGT_8	UGT_8A_1

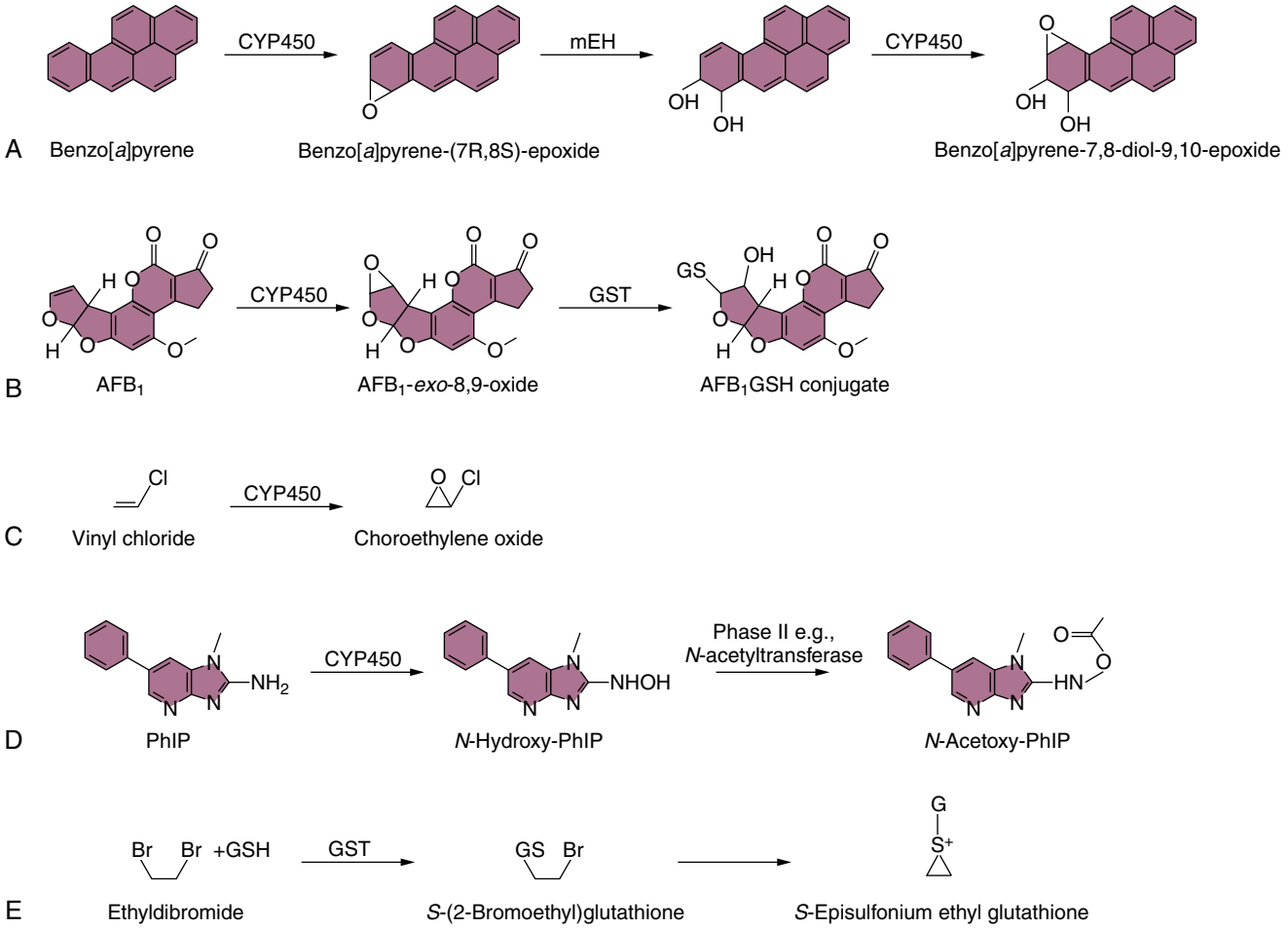


Figure 7-6 Biotransformation either activates or deactivates the ultimate carcinogen **(A)** Sequential action of cytochrome P450 (CYP450) and microsomal epoxide hydrolase (mEH) activates B[*a*]P. **(B)** CYP450 activates while glutathione *S*-transferase (GST)-mediated conjugation of glutathione (GSH) deactivates aflatoxin B_1 (AFB_1). **(C)** Vinyl chloride is activated to its epoxide metabolite by CYP450. **(D)** The 2-amino-1-methyl-6-phenylimidazo-(4,5-*b*)-pyridine (PhIP) is first metabolized by CYP450, then activated by *N*-acetyltransferase. **(E)** GST mediates activation of ethyldibromide.

Vinyl Chloride Biotransformation

Vinyl chloride is the starting material for the production of polyvinyl chloride, used in the fabrication of products such as PVC pipe. The mutagenicity of this liver carcinogen is dependent on metabolic activation by CYP450, and detoxification is mediated by mEH.[18] As shown in Figure 7-6, vinyl chloride is a relatively simple compound. In the presence of oxygen and NADPH, CYP450 2E1 catalyzes formation of a highly unstable epoxide moiety across the central carbon double bond. This epoxide, chloroethylene oxide, is the ultimate carcinogen capable of covalently binding DNA. Chloroethylene oxide can be detoxified via the action of mEH as noted previously or by GST-mediated glutathione conjugation.

Benzidine Biotransformation

Benzidine, an aromatic amine bladder carcinogen, must also undergo metabolic activation to initiate carcinogenesis.[38] CYP450 enzymes catalyze the activation of benzidine via *N*-oxidation. Subsequent to *N*-oxidation, *N*-acetyltransferase (NAT)-catalyzed *O*-acetylation forms electrophilic *N*-acetoxy derivatives capable of attacking DNA. In contrast, *N*-acetylation is also believed to compete with *N*-oxidation and, therefore, is considered a detoxification reaction when it occurs before the formation of the *N*-OH metabolites. *N*-glucuronidation of oxidized benzidine catalyzed by UDP-glucuronosyltransferase (UGT) is a second detoxification mechanism, because *N*-glucuronidation facilitates excretion. Therefore, in the case of benzidine biotransformation, phase II reactions activate and detoxify the carcinogen.

Heterocyclic Amine Biotransformation

Heterocyclic amines, found in cooked meat and fish, are initially activated to genotoxic metabolites via CYP450-mediated oxidation to the *N*-hydroxyl derivative (see Figure 7-6).[42] In particular, this reaction is catalyzed in the liver predominantly by CYP450 1A2 (CYP1A2). The hydroxylated heterocyclic amine metabolites are then further activated by acetyltransferases and sulfotransferases to the ultimate

carcinogen, a highly reactive electrophile. GSTs and UDP-glucuronosyl transferases are thought to deactivate the ultimate carcinogen and permit elimination. Therefore, during the biotransformation of heterocyclic amines, phase II enzymes activate and detoxify the carcinogen.

Ethylene Dibromide (1,2-Dibromoethane) Biotransformation

An additional example of phase II–mediated carcinogen activation is that of the halogenated aliphatic ethyldibromide.[79] Ethylene dibromide is a potent mutagen used as an industrial solvent, gasoline lead scavenger, and fumigant. Following glutathione conjugation of the parent compound, *S*-2-bromoethyl glutathione spontaneously forms an episulfonium ion (see Figure 7-6). This sterically strained molecule is the reactive ultimate carcinogen and primarily attacks the N^7 position of guanine. Again, although GSTs commonly detoxify xenobiotics, glutathione conjugation of ethylene dibromide leads to carcinogen activation.

DNA Repair

A second form of endogenous defense against environmental carcinogenesis is DNA repair. Various forms of carcinogen-induced DNA damage, such as DNA adducts, DNA crosslinks, and double- and single-strand breaks, have been reported following exposure to various carcinogenic insults. To maintain genomic integrity, DNA repair genes have evolved.[80] More than 125 DNA repair enzymes and DNA damage response genes have been identified. The importance of these genes is highlighted by inherited syndromes (e.g., xeroderma pigmentosum [XP], Fanconi anemia, Bloom syndrome, and ataxia-telangiectasia) wherein DNA repair defects render the individual more susceptible to cancer development. These DNA repair proteins can be generally categorized according to the repair pathways in which they function or according to their ability to signal for or regulate DNA repair (see Chapter 4). The predominant human DNA repair pathways include base excision, nucleotide excision, base mismatch, and DNA strand break repair. Simpler, direct repair pathways have also been reported. In cases where DNA damage is excessive, crosstalk may activate apoptotic pathways. In this case, programmed cell death can be viewed as a protective host response.

Defense against Oxidative Stress

Numerous carcinogens, including UV light and heavy metals, are thought to act by inducing oxidative stress and oxidative DNA damage. Endogenous defense systems have evolved to detoxify reactive oxygen species such as hydroxyl radical and superoxide anion. These defense systems range from the free radical scavengers glutathione and vitamin E, to glutathione synthesis enzymes, to antioxidant enzymes such as glutathione transferases, peroxidases, superoxide dismutase and catalase, to DNA repair enzymes that are specialized for repair of oxidative DNA damage.

Host Susceptibility to Environmental Carcinogenesis

As described in the preceding sections, biotransformation of carcinogens, DNA repair, and other cellular events play roles in defending cells against carcinogenic insults. Sequence variation in the genes involved in these cellular processes has been described and is expected, in some cases, to alter cancer risk. In addition, susceptibility to certain carcinogens may vary according to other clinical factors such as developmental stage, disease status, or co-exposure to additional environmental agents. The combined and interacting effects of carcinogen exposure, genetic background, and clinical susceptibility determine the individual's ensuing risk of developing cancer.

Genetic Susceptibility

For years researchers have sought to define the relationship between toxicant exposure outcome and the mediating effects of genetic polymorphisms. Genetic polymorphisms are defined as genetic variations occurring with 1% or greater prevalence in a human population. Polymorphisms can occur in the form of large deletions, small deletions, small insertions, and individual base changes, especially single-nucleotide polymorphisms (SNPs). These polymorphisms can occur in exons, introns, and promoter regions and produce a wide variety of effects ranging from changes in the protein function, to expression-level alterations, to changes in protein stability. The physiologic relevance of genetic variation may be a function of the severity of the alteration and the level of carcinogen exposure. Thus, genetic variation may be most important in the context of low-level exposure, because high-level carcinogen exposure may overwhelm any differences in effects that may result from genetic variation.

Biotransformation Enzyme Polymorphisms and Cancer Risk

Although genetic variation can influence cell signaling, cellular differentiation, apoptosis rate, cellular proliferation rate, or other biochemical processes during chemical carcinogenesis, historically more emphasis has been placed on identification of genetic determinants of carcinogen metabolism and DNA repair. SNPs occur in GST gene exons, introns, and promoter regions, and two-gene deletion polymorphisms have been described.[81] The *GSTM1* and *GSTT1* genes are deleted in about 50% and about 20% to 60% of

the population, respectively. Polymorphisms have also been described for CYP450, NAT, and mEH genes. A number of these alterations have been shown experimentally to affect either the expression level or catalytic activities of their corresponding proteins.[82]

One of the most studied biotransformation enzyme/cancer risk relationships is the relationship between *GSTM1* deletion polymorphism and lung cancer risk. *GSTM1* detoxifies PAHs such as those in cigarette smoke, and meta-analysis of epidemiologic data suggests that *GSTM1* deficiency is a moderate risk factor for lung cancer.[71] Similarly, *GSTM1* deletion may increase the risk of colon and bladder cancers.[72,73] However, some studies of *GSTM1* genotype and cancer phenotype have reported inconsistent outcomes; therefore the relative contribution of this polymorphism requires further investigation. Studies of *GSTM1* deletion polymorphism in the context of other carcinogen-response gene polymorphisms may be critical.

GWAS and Cancer Risk

More recently, genome-wide association studies (GWAS) have largely supplanted single-gene studies as the preferred method for probing the relationship between genetic background, carcinogen exposure, and disease risk.[83] GWAS allow high-throughput scans of the entire genome in cancer case and control groups without prior hypotheses concerning which genes or gene regions may contribute to cancer susceptibility. Numerous groups have attempted to identify the primary genetic determinants of cancer risk using this technique. Their work has revealed that multiple, common alleles each makes a small contribution to the heritability of cancer susceptibility, with no single gene or gene pathway dictating cancer risk.

Clinical Susceptibility

The relatively modest findings derived from GWAS and earlier epidemiologic studies illustrate the complex nature of predicting cancer risk phenotype based on genotype alone. Additional factors such as carcinogen dose and tissue-specific expression of biotransformation genes must be taken into account in the context of low-penetrance phenotypes. In addition, it is well known that developmental stage of the individual, concurrent diseases, co-exposures to other dietary and environmental chemicals, and the frequency, dose, and timing of carcinogen exposure affect the clinical susceptibility to cancer development. Co-exposures to other agents can alter expression or activity of phase I and phase II enzymes and confound the genotype-phenotype relationship. In addition, in case-control and cohort studies, accurate estimates of carcinogen dose are virtually impossible because of recall bias, lack of adequate exposure monitoring technology, effects of dosing frequency or timing, and other confounding factors that influence clinical susceptibility.

Researchers have begun to realize the importance of developmental stage in determining the physiologic response to carcinogen exposure. Accumulating evidence suggests that exposures during the critical periods of organogenesis and tissue differentiation may permanently affect disease risk later in life.[84] Although gene mutation in stem cells or other critical cell populations may contribute, epigenetic reprogramming is believed to be the primary mechanism whereby early life exposures affect health outcomes in childhood or adulthood. During development, epigenetic programs are in constant flux and open to environmental cues. Exposure to agents that affect DNA methylation or histone modification may permanently "reprogram" the response to carcinogens within the affected tissue. The strongest evidence for epigenetic reprogramming of cancer susceptibility comes from studies of reproductive tract and breast cancers. As an example, in utero exposure to the xenoestrogen DES is associated with a significantly increased risk of vaginal clear-cell carcinoma. Studies in animal models show that DES exposure alters DNA methylation patterns. Therefore, the stage at which an individual is exposed to environmental agents could be a critical determinant of susceptibility. Even a brief exposure to epigenetic reprogramming agents during development could potentially alter host susceptibility to carcinogenesis. Despite these inherent challenges in assessing co-exposures, temporal effects, and multiple genetic factors, the ultimate goal is to achieve cancer risk modeling that takes into account both inheritance of polymorphisms in genes encoding carcinogen defense pathways and other clinical factors that affect susceptibility.

Cancer Prevention

Because a significant fraction of cancer risk appears to be attributable to environmental factors, cancer prevention should be an attainable goal. Multiple approaches to cancer prevention have been proposed and include chemoprevention and, more simply, exposure reduction. As new products and pollutants are introduced into the environment, vigilance in hazard identification should largely prevent population-wide health crises such as those that led to the discovery of many occupational carcinogens in the 1970s and earlier. In addition, careful analysis of current dietary and other environmental exposures will increase the understanding of existing hazards. Finally, understanding of the underlying molecular mechanisms associated with the carcinogenic process will allow for the design of effective chemoprevention strategies.

Hazard Identification

Assays

An important aspect of cancer prevention is hazard identification. To effectively prevent human exposure to carcinogens, the carcinogen must be recognized as such. Hazard identification occurs via multiple avenues under the direction of numerous institutions. Academic institutes, corporations, and government agencies all contribute to the identification of carcinogenic agents. Initial screening is often conducted using short-term, in vitro techniques. Several widely used assays have been developed and measure the mutagenicity of suspected carcinogens.

Ames Assay

The Ames assay of mutagenicity utilizes *Salmonella typhimurium* bacterial strains with unique growth requirements to detect mutagenicity of test compounds.[85] In these assays, histidine-synthesis–deficient *Salmonella* strains are initially grown in the presence of exogenous histidine and are subsequently exposed to test compounds. Mutations in histidine-synthesis genes revert the bacterial strain to a histidine-independent status, which can be detected by growth in minimal-histidine media. Only those bacteria that have acquired specific mutations in histidine-synthesis genes are able to form colonies. Because bacterial strains cannot activate procarcinogens via CYP450 biotransformation, inclusion of mammalian metabolic enzymes is an important feature of this "reversion" assay.

HPRT Assay

The hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay uses cultured human somatic cells to detect mutagenic agents. The normal function of HPRT in cells is to recycle nucleotide bases from degraded DNA. To detect mutations in the HPRT gene, cells are first exposed to the test compound and then exposed to a toxic nucleotide analogue, 6-thioguanine (6TG). When HPRT is nonmutated and functioning, 6TG is incorporated into DNA, triggering cell death. However, when HPRT is inactivated by mutations, no 6TG is incorporated, and the cells remain viable. Therefore, the number of surviving cells after a defined period of cell growth following 6TG exposure reflects the mutagenicity of the test agent.

Additional In Vitro Carcinogen Identification Assays

In addition to the HPRT and Ames assays, several other direct and indirect in vitro assays for the detection of genetic damage have been developed. Assays of changes at the chromosomal level in human cells include (1) the chromosome aberration assay, wherein metaphase chromosomes are examined for abnormalities; (2) the sister chromatid exchange (SCE) assay, wherein exchanges of identical pieces of chromosomes in duplicated sister chromatids are examined in metaphase cells; (3) the micronucleus assay, wherein the number of chromosome fragments referred to as *micronuclei* are counted; and (4) the comet (or single-cell gel electrophoresis) assay, wherein DNA strand breaks in individual cells are visualized using fluorescence microscopy.

In Vivo Assays

Two-year bioassays in rodents are currently used extensively for carcinogen identification. Whole-animal assays are conducted to determine the carcinogenic potential of an agent when delivered over the lifespan, in a more physiologically relevant model. Of the approximately 200 agents classified as human carcinogens, almost all have been shown to cause cancer in rats or mice, highlighting the utility of animal studies in the identification of carcinogens. Rodents are administered the test compound via the exposure route most relevant to human scenarios at two doses: the maximum tolerated dose (MTD) and one half the MTD. The compound is administered for a majority of the lifespan of the animal, and tumor incidence at all sites is recorded. Generally, the rat is recommended for the first 2-year carcinogenicity study. These data are then supplemented with additional short- or medium-term in vivo studies or with a 2-year carcinogenicity study in another rodent species such as the mouse. Short- and medium-term testing may include the use of transgenic or "knockout" mouse models wherein an oncogene is overexpressed or a tumor suppressor gene allele is missing, although the validity of using these genetically altered models is still under debate.

The costly nature of in vivo screens (more than 800 rodents and histopathological analysis of more than 40 tissues) limits their utility.[86] Only approximately 1500 chemicals have been adequately examined. Recent effort has been directed toward developing predictive models for more thorough or higher throughput screening of new drugs and other agents. For example, the vast majority of known carcinogens is mutagenic or genotoxic; therefore, several groups have proposed an expanded battery of DNA-based tests, including tests of DNA adduct formation, DNA strand breaks, and DNA repair. In addition, toxicogenomic strategies for the identification of gene expression profiles that are predictive of genotoxic and non-genotoxic carcinogenicity have been proposed.

Non-genotoxic Carcinogens

The identification and analysis of non-genotoxic carcinogens are less straightforward than are those for genotoxic agents. These agents are identified in the context of the 2-year rodent bioassay. Agents that are identified as carcinogenic in these in vivo assays but do not directly interact with DNA

are classified as non-genotoxic carcinogens.[87] Non-genotoxic carcinogens characteristically induce tumors in only one or a few species and only after a threshold dose is achieved. Non-genotoxic carcinogens are not detected in in vitro assays of mutagenicity such as the Ames or HPRT assays. Many of these non-genotoxic carcinogens possess properties similar to tumor promoters, suggesting, together with a lack of genotoxicity, that they work mechanistically differently from classical, genotoxic carcinogens. Considerable debate is ongoing concerning the best way to identify and regulate such compounds (see subsequent sections).

Risk Assessment and Regulation of Carcinogen Exposure

As carcinogen exposure scenarios are identified, an assessment of predicted exposure dose and the expected degree of health hazard is conducted. This informs an estimation of overall cancer risk associated with the observed exposure. Estimating cancer risk helps investigators determine when and if behavior modifications should be enforced. This process of predicting cancer risk in a given exposure scenario is referred to as *risk assessment*, whereas the response to predicted risk is referred to as *risk management*. The EPA is responsible for risk assessment in areas of known or suspected exposure of the population to carcinogens and makes recommendations for risk management to minimize health consequences due to environmental contamination.

Risk assessment concerning mutagenic carcinogen exposure assumes that no threshold dose exists. That is, no safe exposure level can be identified because any exposure dose could, in theory, induce a mutation in a critical target gene, thereby elevating cancer risk. Extrapolation of a safe level of human exposure to non-genotoxic carcinogens is more complex and requires multiple assumptions. For instance, it is assumed that an agent found to be a non-genotoxic carcinogen in rodents would be toxic to humans and that the no observable adverse effect level (NOAEL) in rodents could be applied to humans. Such decisions are greatly enhanced by mechanistic information so that judgments can be made concerning potential threat to human health. In 2005, the EPA released new risk assessment guidelines that acknowledge the different mode of action for non-genotoxic carcinogens and provide for this deviation during risk-management decision making.

Prevention Strategies

The goal of risk assessment and risk management is to prevent cancer by anticipating and circumventing carcinogen exposure. However, the etiology of certain cancers is still unknown. In many cases, risk assessment is impossible or risk management measures are unavailable. Furthermore, some carcinogen exposures are unavoidable, or avoidance is not practically feasible. For instance, therapeutic radiation and certain chemotherapy drugs are known carcinogens; however, the risk-to-benefit ratio still favors voluntary exposure, despite health risk. In these instances, prevention tactics are needed to counteract the carcinogenic process, especially in the absence of effective treatment options. Several approaches to prevention have been taken in recent years with varying degrees of promise.[88]

Vaccination

Vaccination is among the most promising of approaches for biologic carcinogens such as HBV, HPV, and *Helicobacter pylori*.[89] The development of vaccines to block initial infection with carcinogenic bacteria or virus would presumably prevent or reduce associated cancers. As an example, HPV vaccines have been developed to limit the spread of the virus and reduce the incidence of cervical cancer. In addition to this traditional use of vaccination, the use of vaccines against oncoantigens has also been proposed to prevent cancer via stimulating immune mechanisms to attack small cancerous lesions. Oncoantigens, which are tumor-associated molecules, are used to stimulate persistent immune memory mechanisms. When the antigen is later detected via immune surveillance, an effective adaptive immune response is mounted. In theory, the immune system is primed to detect and destroy any cancer cells expressing the oncoantigen. The success of vaccines in the prevention of tumors in animal models has been documented; however, the utility of such vaccines to prevent human tumors must still be validated.

Chemoprevention

Chemoprevention strategies for reducing the incidence of cancer have also been proposed. For instance, chemicals that upregulate biotransformation enzymes (in particular, phase II enzymes) have been investigated as chemopreventive agents.[75,90] Because most phase II biotransformation reactions reduce chemical reactivity of the parent compound, the rationale for inducing phase II enzymes or their cofactors is to reduce the mutagenicity of initiating agents. For instance, oltipraz administration has been shown to attenuate AFB_1 toxicity in rats. Oltipraz elevates GST activity, likely via activation of antioxidant response elements within GST promoter regions. Oltipraz may also inhibit the activation of aflatoxin by CYP450. The challenge associated with enzyme induction as a chemopreventive approach is that not all phase II biotransformation reactions are detoxification reactions. Because humans are exposed to a wide variety of carcinogens, induction of biotransformation enzymes may

be simultaneously beneficial and detrimental. Therefore, the decision to use of this type of chemopreventive agent must weigh multiple factors such as carcinogen target organ, agent distribution, and exposure scenario.

In addition to agents that induce the expression of detoxification enzymes, agents that combat or prevent oxidative stress are potential chemopreventive agents. Oxidative stress is believed to contribute to the formation of multiple cancer types; consequently, treatment with antioxidant agents may block carcinogenesis. In this regard, selenium, α-tocopherol, EGCG, and lycopene are potent antioxidants under study for chemopreventive properties. Similarly, inflammation is believed to contribute to formation of numerous cancer types. Agents such as cyclooxygenase-2 (COX-2) inhibitors (i.e., celecoxib) as well as other nonsteroidal antiinflammatory drugs (NSAIDs) have been proposed as chemopreventive agents to combat procarcinogenic inflammation. In addition, hormonal agents have been proposed for the chemoprevention of cancers of reproductive organs such as breast and prostate cancers.[91] For instance, selective estrogen receptor modulators (SERMs) have been proposed to prevent breast cancer by blocking the action of procarcinogenic estrogen. Tamoxifen, an antiestrogenic agent, was first approved for the treatment of advanced breast cancer but also reduced the risk of contralateral breast cancer occurrence. Tamoxifen has since been approved as a chemopreventive agent in high-risk patients.

Summary and Conclusions

Cancer is known to develop over many years and is determined by the interaction of host genetic factors as well as environmental exposures. Environmental factors appear to play a major role in determining cancer risk. Of the known cancer risk factors, smoking and diet account for a significant proportion of cancer deaths. There are many types of environmental carcinogens including biologic agents (e.g., viruses), chemicals (e.g., PAH), and physical agents (e.g., solar radiation). The linkage between environmental exposure and cancer in humans is strong in some cases (e.g., asbestos and mesothelioma of the lung), whereas in other cases the environmental etiologic factors are less well understood (e.g., breast and prostate cancers). Epidemiology studies, together with studies in model systems, especially animal model systems, provide the evidence used to determine the relative risk of specific environmental exposures. Categorizing cancer risk from environmental agents is an ongoing process conducted by the NTP and the IARC. Study of genetic polymorphisms in various genes involved in the carcinogenic process is leading to a better understanding of the overall risk associated with environmental exposures and identification of high-risk populations to target prevention strategies. Research on the underlying mechanisms associated with environmental carcinogenesis provides the basis for early detection and identification of target molecules for chemoprevention and/or intervention strategies. Carcinogens are known to target oncogenes and tumor suppressor genes through DNA damage and/or to alter cellular signaling pathways in bringing about the changes associated with cancer formation in specific tissues. Ultimately, environmental carcinogenesis occurs via the stepwise accumulation of genetic alterations leading to invasive and metastatic lesions. Finally, although many regulatory mechanisms exist to protect the public, diligence is required to guard them from future unintended carcinogen exposures. It will remain prudent to closely monitor the environment for potential human carcinogens.

Key References

1. Luch A. Nature and nurture—lessons from chemical carcinogenesis. *Nat Rev Cancer*. 2005;5:113-125.
2. Harvey RG. Historical overview of chemical carcinogenesis. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:1-26.
4. Jemal A, et al. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev*. 2010;19(8):1893-1907.
6. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. *CA Cancer J Clin*. 2009;59(6):366-378.
7. Chang S, Bondy ML, Gurney JG. Cancer epidemiology. In: Pollock RE, ed. *UICC Manual of Clinical Oncology*. New York, NY: John Wiley & Sons, Inc; 2004:91-120.
9. Hecht SS. Progress and challenges in selected areas of tobacco carcinogenesis. *Chem Res Toxicol*. 2008;21(1):160-171.
10. Hecht S. Tobacco smoke carcinogens and lung cancer. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:53-74.
11. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer*. 2011;11(12):886-895.
12. Hursting SD, Digiovanni J, Dannenberg AJ, et al. Obesity, energy balance, and cancer: new opportunities for prevention. *Cancer Prev Res (Phila)*. 2012;5(11):1260-1272.
14. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid Redox Signal*. 2008;10(3):475-510.
18. Sherman M. Vinyl chloride and the liver. *J Hepatol*. 2009;51(6):1074-1081.
19. President's Cancer Panel. *Environmental Factors in Cancer*. Indianapolis, Ind. Bethesda, Md: Dept. of Health & Human Services National Institutes of Health, National Cancer Institute; October 21, 2008.
22. Narayanan DL, Saladi RN, Fox JL. Ultraviolet radiation and skin cancer. *Int J Dermatol*. 2010;49(9):978-986.
25. de Gruijl FR, Rebel H. Early events in UV carcinogenesis—DNA damage, target cells and mutant p53 foci. *Photochem Photobiol*. 2008;84(2):382-387.
26. Flaherty KT, Hodi FS, Fisher DE. From genes to drugs: targeted strategies for melanoma. *Nat Rev Cancer*. 2012;12(5):349-361.
27. Wakeford R. The cancer epidemiology of radiation. *Oncogene*. 2004;23(38):6404-6428.

32. McHale CM, Zhang L, Smith MT. Current understanding of the mechanism of benzene-induced leukemia in humans: implications for risk assessment. *Carcinogenesis*. 2012;33(2):240-252.
33. Broyde S, Wang L, Cai Y, et al. Covalent polycyclic aromatic hydrocarbon-DNA adducts: carcinogenicity, structure and function. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:181-208.
35. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120(Suppl 1):S28-S48.
36. Groopman JD, Wogan GN. Aflatoxin and hepatocellular carcinoma. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:113-134.
37. Golka K, Kopps S, Myslak ZW. Carcinogenicity of azo colorants: influence of solubility and bioavailability. *Toxicol Lett*. 2004;151(1):203-210.
41. Knize MG, Felton JS. Formation and human risk of carcinogenic heterocyclic amines formed from natural precursors in meat. *Nutr Rev*. 2005;63(5):158-165.
42. Cross AJ, Sinha R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ Mol Mutagen*. 2004;44(1):44-55.
44. Gordon T, Bowser D. Beryllium: genotoxicity and carcinogenicity. *Mutat Res*. 2003;533(1-2):99-105.
45. Boffetta P, Fryzek JP, Mandel JS. Occupational exposure to beryllium and cancer risk: a review of the epidemiologic evidence. *Crit Rev Toxicol*. 2012;42(2):107-118.
46. Hollins DM, McKinley MA, Williams C, et al. Beryllium and lung cancer: a weight of evidence evaluation of the toxicological and epidemiological literature. *Crit Rev Toxicol*. 2009;39(Suppl 1):1-32.
47. Joseph P. Mechanisms of cadmium carcinogenesis. *Toxicol Appl Pharmacol*. 2009;238(3):272-279.
48. Tapio S, Grosche B. Arsenic in the aetiology of cancer. *Mutat Res*. 2006;612(3):215-246.
49. Some drinking-water disinfectants and contaminants, including arsenic. *IARC Monogr Eval Carcinog Risks Hum*. 2004;84:1-477.
50. Huang C, Ke Q, Costa M, Shi X. Molecular mechanisms of arsenic carcinogenesis. *Mol Cell Biochem*. 2004;255(1-2):57-66.
51. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. *Environ Health Perspect*. 2011;119(1):11-19.
52. Zhitkovich A. Chromium in drinking water: sources, metabolism, and cancer risks. *Chem Res Toxicol*. 2011;24(10):1617-1629.
54. Robinson BW, Musk AW, Lake RA. Malignant mesothelioma. *Lancet*. 2005;366(9483):397-408.
55. Carbone M, Yang H. Molecular pathways: targeting mechanisms of asbestos and erionite carcinogenesis in mesothelioma. *Clin Cancer Res*. 2012;18(3):598-604.
57. Dunn BK, Wickerham DL, Ford LG. Prevention of hormone-related cancers: breast cancer. *J Clin Oncol*. 2005;23(2):357-367.
58. Parnes HL, Thompson IM, Ford LG. Prevention of hormone-related cancers: prostate cancer. *J Clin Oncol*. 2005;23(2):368-377.
59. Abel EL, DiGiovanni J. Multistage carcinogenesis. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:27-52.
60. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
61. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc*. 2009;4(9):1350-1362.
63. Rho O, Kim DJ, Kiguchi K, Digiovanni J. Growth factor signaling pathways as targets for prevention of epithelial carcinogenesis. *Mol Carcinog*. 2011;50(4):264-279.
64. Chan KS, Sano S, Kiguchi K, et al. Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. *J Clin Invest*. 2004;114(5):720-728.
65. Wilker E, Lu J, Rho O, Carbajal S, Beltrán L, DiGiovanni J. Role of PI3K/Akt signaling in insulin-like growth factor-1 (IGF-1) skin tumor promotion. *Mol Carcinog*. 2005;44(2):137-145.
67. Vineis P, Schatzkin A, Potter JD. Models of carcinogenesis: an overview. *Carcinogenesis*. 2010;31(10):1703-1709.
68. Ziech D, Franco R, Pappa A, et al. The role of epigenetics in environmental and occupational carcinogenesis. *Chem Biol Interact*. 2010;188(2):340-349.
69. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis*. 2007;22(2):91-103.
73. Olivier M, Hollstein M, Hainaut P. TP53 Mutations in human cancers: origins, consequences and clinical use. In: Levine A, Lane D, eds. *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2012:1-17.
75. Yates MS, Kensler TW. Detoxification of chemical carcinogens and chemoprevention. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:159-180.
76. Kleiner HE, Vulimiri SV, Hatten WB, et al. Role of cytochrome p4501 family members in the metabolic activation of polycyclic aromatic hydrocarbons in mouse epidermis. *Chem Res Toxicol*. 2004;17(12):1667-1674.
80. Lange SS, Takata K, Wood RD. DNA polymerases and cancer. *Nat Rev Cancer*. 2011;11(2):96-110.
81. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. *Oncogene*. 2006;25(11):1639-1648.
82. Singh MS, Michael M. Role of xenobiotic metabolic enzymes in cancer epidemiology. *Methods Mol Biol*. 2009;472:243-264.
83. Chung CC, Chanock SJ. Current status of genome-wide association studies in cancer. *Hum Genet*. 2011;130(1):59-78.
84. Walker CL, Ho SM. Developmental reprogramming of cancer susceptibility. *Nat Rev Cancer*. 2012;12(7):479-486.
86. Waters MD, Jackson M, Lea I. Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods. *Mutat Res*. 2010;705(3):184-200.
88. Lippman SM, Hawk ET. Cancer prevention: from 1727 to milestones of the past 100 years. *Cancer Res*. 2009;69(13):5269-5284.
89. Lollini PL, Cavallo F, Nanni P, Forni G. Vaccines for tumour prevention. *Nat Rev Cancer*. 2006;6(3):204-216.
90. Pool-Zobel B, Veeriah S, Bohmer FD. Modulation of xenobiotic metabolising enzymes by anticarcinogens—focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutat Res*. 2005;591(1-2):74-92.
91. Neill MG, Fleshner NE. An update on chemoprevention strategies in prostate cancer for 2006. *Curr Opin Urol*. 2006;16(3):132-137.

References

1. Luch A. Nature and nurture—lessons from chemical carcinogenesis. *Nat Rev Cancer*. 2005;5:113-125.
2. Harvey RG. Historical overview of chemical carcinogenesis. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:1-26.
3. Kennaway E. The identification of a carcinogenic compound in coal-tar. *Br Med J*. 1955;2(4942):749-752.
4. Jemal A, et al. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev*. 2010;19(8):1893-1907.
5. Ziegler RG, Hoover RN, Pike NC, et al. Migration patterns and breast cancer risk in Asian-American women. *J Natl Cancer Inst*. 1993;85(22):1819-1827.
6. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. *CA Cancer J Clin*. 2009;59(6):366-378.
7. Chang S, Bondy ML, Gurney JG. Cancer epidemiology. In: Pollock RE, ed. *UICC Manual of Clinical Oncology*. New York, NY: John Wiley & Sons, Inc; 2004:91-120.
8. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst*. 1981;66(6):1191-1308.
9. Hecht SS. Progress and challenges in selected areas of tobacco carcinogenesis. *Chem Res Toxicol*. 2008;21(1):160-171.
10. Hecht S. Tobacco smoke carcinogens and lung cancer. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:53-74.
11. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. *Nat Rev Cancer*. 2011;11(12):886-895.
12. Hursting SD, Digiovanni J, Dannenberg AJ, et al. Obesity, energy balance, and cancer: new opportunities for prevention. *Cancer Prev Res (Phila)*. 2012;5(11):1260-1272.
13. Montesano R, Hall J. Environmental causes of human cancers. *Eur J Cancer*. 2001;37(Suppl 8):S67-S87.
14. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid Redox Signal*. 2008;10(3):475-510.
15. Nortier JL, Martinez MC, Schmiser HH, et al. Urothelial carcinoma associated with the use of a Chinese herb (*Aristolochia fangchi*). *N Engl J Med*. 2000;342(23):1686-1692.
16. Garte SJ. Ionizing radiation as a carcinogen. In: Sipes G, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. New York, NY: Elsevier Science, Ltd; 1997:281-302.
17. Delclos KB, Kadlubar FF. Carcinogenic aromatic amines and amides. In: Sipes G, McQueen CA, Gandolfi AJ, eds. *Comprehensive Toxicology*. New York, NY: Elsevier Science, Ltd; 1997:141-170.
18. Sherman M. Vinyl chloride and the liver. *J Hepatol*. 2009;51(6):1074-1081.
19. President's Cancer Panel. *Environmental Factors in Cancer*. Indianapolis, Ind. Bethesda, Md: Dept. of Health & Human Services National Institutes of Health, National Cancer Institute; October 21, 2008.
20. Pitot HCI, Dragan YP. Chemical carcinogenesis. In: Klaassen CD, ed. *Casarett & Doull's Toxicology, The Basic Science of Poisons*. New York, NY: McGraw-Hill; 1996:201-267.
21. Sipes G, McQueen CA, Gandolfi AJ. Comprehensive toxicology. In: Bowden GT, Fischer SM, eds. *Chemical Carcinogens and Anticarcinogens*. Vol 12. New York, NY: Elsevier Science Inc; 1997.
22. Narayanan DL, Saladi RN, Fox JL. Ultraviolet radiation and skin cancer. *Int J Dermatol*. 2010;49(9):978-986.
23. de Laat A, van Tilburg M, van der Leun JC, van Vloten WA, de Gruijl FR. Cell cycle kinetics following UVA irradiation in comparison to UVB and UVC irradiation. *Photochem Photobiol*. 1996;63(4):492-497.
24. Anderson RR, Parrish JA. The optics of human skin. *J Invest Dermatol*. 1981;77(1):13-19.
25. de Gruijl FR, Rebel H. Early events in UV carcinogenesis—DNA damage, target cells and mutant p53 foci. *Photochem Photobiol*. 2008;84(2):382-387.
26. Flaherty KT, Hodi FS, Fisher DE. From genes to drugs: targeted strategies for melanoma. *Nat Rev Cancer*. 2012;12(5):349-361.
27. Wakeford R. The cancer epidemiology of radiation. *Oncogene*. 2004;23(38):6404-6428.
28. Little JB. Radiation carcinogenesis. *Carcinogenesis*. 2000;21(3):397-404.
29. Little JB. What are the risks of low-level exposure to alpha radiation from radon? *Proc Natl Acad Sci U S A*. 1997;94(12):5996-5997.
30. Dipple A. Polycyclic aromatic hydrocarbon carcinogenesis. In: Harvey RG, ed. *Polycyclic Hydrocarbons and Carcinogenesis. ACS Symposium Series 283*. Philadelphia, Pa: American Chemical Society; 1985:1-17.
31. Duarte-Davidson R, Courage C, Rushton L, Levy L. Benzene in the environment: an assessment of the potential risks to the health of the population. *Occup Environ Med*. 2001;58(1):2-13.
32. McHale CM, Zhang L, Smith MT. Current understanding of the mechanism of benzene-induced leukemia in humans: implications for risk assessment. *Carcinogenesis*. 2012;33(2):240-252.
33. Broyde S, Wang L, Cai Y, et al. Covalent polycyclic aromatic hydrocarbon-DNA adducts: carcinogenicity, structure and function. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:181-208.
34. Rubin H. Synergistic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: a bio-historical perspective with updates. *Carcinogenesis*. 2001;22(12):1903-1930.
35. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol Sci*. 2011;120(Suppl 1):S28-S48.
36. Groopman JD, Wogan GN. Aflatoxin and hepatocellular carcinoma. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:113-134.
37. Golka K, Kopps S, Myslak ZW. Carcinogenicity of azo colorants: influence of solubility and bioavailability. *Toxicol Lett*. 2004;151(1):203-210.
38. Zenser TV, Lakshmi VM, Hsu FF, Davis BB. Metabolism of *N*-acetylbenzidine and initiation of bladder cancer. *Mutat Res*. 2002;506-507:29-40.
39. Whysner J, Verna L, Williams GM. Benzidine mechanistic data and risk assessment: species- and organ-specific metabolic activation. *Pharmacol Ther*. 1996;71(1-2):107-126.
40. Knekt P, Järvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastrointestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: a follow-up study. *Int J Cancer*. 1999;80(6):852-856.
41. Knize MG, Felton JS. Formation and human risk of carcinogenic heterocyclic amines formed from natural precursors in meat. *Nutr Rev*. 2005;63(5):158-165.
42. Cross AJ, Sinha R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ Mol Mutagen*. 2004;44(1):44-55.
43. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem Res Toxicol*. 1998;11(6):559-603.
44. Gordon T, Bowser D. Beryllium: genotoxicity and carcinogenicity. *Mutat Res*. 2003;533(1-2):99-105.
45. Boffetta P, Fryzek JP, Mandel JS. Occupational exposure to beryllium and cancer risk: a review of the epidemiologic evidence. *Crit Rev Toxicol*. 2012;42(2):107-118.
46. Hollins DM, McKinley MA, Williams C, et al. Beryllium and lung cancer: a weight of evidence evaluation of the toxicological and epidemiological literature. *Crit Rev Toxicol*. 2009;39(Suppl 1):1-32.
47. Joseph P. Mechanisms of cadmium carcinogenesis. *Toxicol Appl Pharmacol*. 2009;238(3):272-279.
48. Tapio S, Grosche B. Arsenic in the aetiology of cancer. *Mutat Res*. 2006;612(3):215-246.
49. Some drinking-water disinfectants and contaminants, including arsenic. *IARC Monogr Eval Carcinog Risks Hum*. 2004;84:1-477.
50. Huang C, Ke Q, Costa M, Shi X. Molecular mechanisms of arsenic carcinogenesis. *Mol Cell Biochem*. 2004;255(1-2):57-66.
51. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. *Environ Health Perspect*. 2011;119(1):11-19.
52. Zhitkovich A. Chromium in drinking water: sources, metabolism, and cancer risks. *Chem Res Toxicol*. 2011;24(10):1617-1629.
53. Ding M, Shi X. Molecular mechanisms of Cr(VI)-induced carcinogenesis. *Mol Cell Biochem*. 2002;234-235(1-2):293-300.
54. Robinson BW, Musk AW, Lake RA. Malignant mesothelioma. *Lancet*. 2005;366(9483):397-408.
55. Carbone M, Yang H. Molecular pathways: targeting mechanisms of asbestos and erionite carcinogenesis in mesothelioma. *Clin Cancer Res*. 2012;18(3):598-604.
56. Amre DK, Infante-Rivard C, Dufresne A, Durgawale PM, Ernst P. Case-control study of lung cancer among sugar cane farmers in India. *Occup Environ Med*. 1999;56(8):548-552.

57. Dunn BK, Wickerham DL, Ford LG. Prevention of hormone-related cancers: breast cancer. *J Clin Oncol*. 2005;23(2):357-367.
58. Parnes HL, Thompson IM, Ford LG. Prevention of hormone-related cancers: prostate cancer. *J Clin Oncol*. 2005;23(2):368-377.
59. Abel EL, DiGiovanni J. Multistage carcinogenesis. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:27-52.
60. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
61. Abel EL, Angel JM, Kiguchi K, DiGiovanni J. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc*. 2009;4(9):1350-1362.
62. Brookes P, Lawley PD. Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin: relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. *Nature*. 1964;202:781-784.
63. Rho O, Kim DJ, Kiguchi K, Digiovanni J. Growth factor signaling pathways as targets for prevention of epithelial carcinogenesis. *Mol Carcinog*. 2011;50(4):264-279.
64. Chan KS, Sano S, Kiguchi K, et al. Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. *J Clin Invest*. 2004;114(5):720-728.
65. Wilker E, Lu J, Rho O, Carbajal S, Beltrán L, DiGiovanni J. Role of PI3K/Akt signaling in insulin-like growth factor-1 (IGF-1) skin tumor promotion. *Mol Carcinog*. 2005;44(2):137-145.
66. Slaga TJ. Mechanisms of tumor promotion. In: Slaga TJ, ed. *Tumor Promotion in Internal Organs*. Vol. 1. Boca Raton, Fla: CRC Press, Inc; 1983.
67. Vineis P, Schatzkin A, Potter JD. Models of carcinogenesis: an overview. *Carcinogenesis*. 2010;31(10):1703-1709.
68. Ziech D, Franco R, Pappa A, et al. The role of epigenetics in environmental and occupational carcinogenesis. *Chem Biol Interact*. 2010;188(2):340-349.
69. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis*. 2007;22(2):91-103.
70. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. *Nat Rev Cancer*. 2002;2(5):331-341.
71. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell*. 1996;87(2):159-170.
72. Wistuba II, Behrens C, Milchgrub S, et al. Sequential molecular abnormalities are involved in the multistage development of squamous cell lung carcinoma. *Oncogene*. 1999;18(3):643-650.
73. Olivier M, Hollstein M, Hainaut P. TP53 Mutations in human cancers: origins, consequences and clinical use. In: Levine A, Lane D, eds. *Cold Spring Harbor Perspectives in Biology*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2012:1-17.
74. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science*. 1996;274(5286):430-432.
75. Yates MS, Kensler TW. Detoxification of chemical carcinogens and chemoprevention. In: Penning TM, ed. *Chemical Carcinogenesis*. New York, NY: Springer; 2011:159-180.
76. Kleiner HE, Vulimiri SV, Hatten WB, et al. Role of cytochrome p4501 family members in the metabolic activation of polycyclic aromatic hydrocarbons in mouse epidermis. *Chem Res Toxicol*. 2004;17(12):1667-1674.
77. Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol Sci*. 1999;49(2):156-164.
78. Van Ness KP, McHugh TE, Bammler TK, Eaton DL. Identification of amino acid residues essential for high aflatoxin B1-8,9-epoxide conjugation activity in alpha class glutathione S-transferases through site-directed mutagenesis. *Toxicol Appl Pharmacol*. 1998;152(1):166-174.
79. Ozawa N, Guengerich FP. Evidence for formation of an S-[2-(N7-guanyl) ethyl]glutathione adduct in glutathione-mediated binding of the carcinogen 1,2-dibromoethane to DNA. *Proc Natl Acad Sci U S A*. 1983;80(17):5266-5270.
80. Lange SS, Takata K, Wood RD. DNA polymerases and cancer. *Nat Rev Cancer*. 2011;11(2):96-110.
81. McIlwain CC, Townsend DM, Tew KD. Glutathione S-transferase polymorphisms: cancer incidence and therapy. *Oncogene*. 2006;25(11):1639-1648.
82. Singh MS, Michael M. Role of xenobiotic metabolic enzymes in cancer epidemiology. *Methods Mol Biol*. 2009;472:243-264.
83. Chung CC, Chanock SJ. Current status of genome-wide association studies in cancer. *Hum Genet*. 2011;130(1):59-78.
84. Walker CL, Ho SM. Developmental reprogramming of cancer susceptibility. *Nat Rev Cancer*. 2012;12(7):479-486.
85. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res*. 2000;455(1-2):29-60.
86. Waters MD, Jackson M, Lea I. Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods. *Mutat Res*. 2010;705(3):184-200.
87. Silva Lima B, Van der Laan JW. Mechanisms of nongenotoxic carcinogenesis and assessment of the human hazard. *Regul Toxicol Pharmacol*. 2000;32(2):135-143.
88. Lippman SM, Hawk ET. Cancer prevention: from 1727 to milestones of the past 100 years. *Cancer Res*. 2009;69(13):5269-5284.
89. Lollini PL, Cavallo F, Nanni P, Forni G. Vaccines for tumour prevention. *Nat Rev Cancer*. 2006;6(3):204-216.
90. Pool-Zobel B, Veeriah S, Bohmer FD. Modulation of xenobiotic metabolising enzymes by anticarcinogens—focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. *Mutat Res*. 2005;591(1-2):74-92.
91. Neill MG, Fleshner NE. An update on chemoprevention strategies in prostate cancer for 2006. *Curr Opin Urol*. 2006;16(3):132-137.

Elspeth Payne and A. Thomas Look

8

Animal Models: Flies, Fish, and Yeast

The molecular pathogenesis of human cancer is a complex process that often requires the cooperation of genetic mutations within many cellular pathways that ultimately lead to tumorigenesis. Simple model organisms with conserved genes and developmental pathways offer systems with which to dissect the role of individual genes and their contribution to the development of cancer in vivo. From single-celled yeasts to vertebrate fish such as the zebrafish, each model system provides its own unique strengths with which to identify new genes and to elucidate genetic interactions required for the development of cancer.

Why Use a Simple Model Organism?

Cancer develops as a result of disruption of the normal physiological processes of cell growth, differentiation, and proliferation. Genes involved in these processes encode transcription factors and other regulatory proteins controlling the cell cycle, apoptosis, and survival. Many of these genes, found in the simplest eukaryotes, are conserved with higher species. Over several decades the development of tools for forward genetic analysis based on phenotype in simple organisms has led to yeasts *(Saccharomyces cerevisiae)*, the fruit fly *(Drosophila melanogaster)*, and more recently the zebrafish *(Danio rerio)* emerging as the key simple organisms for investigating cancer genetics (Figure 8-1).

In addition to their individual strengths for investigating conserved pathways there are three main practical reasons to use a simple model organism: time, space, and tractability. Yeasts such as *Saccharomyces cerevisiae (S. cerevisiae)* and *Schizosaccharomyces pombe (S. pombe)* are single-cell organisms. They have comparatively few genes and little intronic DNA. They replicate rapidly by budding *(S. cerevisiae)* or fission *(S. pombe)* and can be maintained in large numbers in both haploid and diploid states, facilitating the isolation and investigation of recessive mutations. Yeast cell numbers double every 100 minutes (given adequate nutrition), and these organisms are safe and cheap to maintain. The whole organism can be readily visualized by light microscopy, and the incorporation of fluorescent proteins allows subcellular localization of specific proteins in real time. Similarly, *Drosophila melanogaster* has a life cycle of 10 days, and large numbers of animals can be maintained in a small space. This multicellular organism can be used to examine cell-cell interactions and the roles of non-cell autonomous gene function in the development of cancer. Although *Drosophila melanogaster* does not develop cancer in its classical form and lacks the closed blood system of vertebrates, ingenious genetic techniques have been applied to model pathways involved in the development of cancer and invasive metastases in this organism, which has the added advantage of being both multicellular and tractable to single-cell resolution.[1] The zebrafish is relatively new to the field of well-characterized model organisms but has rapidly gained popularity. As a vertebrate with a closed vascular system (and beating heart), it provides an ideal intermediate model system between studies in invertebrates and small mammals such as the mouse. In contrast to the mouse, zebrafish development occurs rapidly outside the mother in transparent embryos, allowing direct visualization of the evolving systems and easy analysis of incorporated fluorescent markers. Its small size and high fecundity allow it to be easily maintained. The ability to directly visualize development in the embryo is particularly beneficial given that proto-oncogenes often have a crucial role during embryonic development.

Genetic Conservation and Synteny

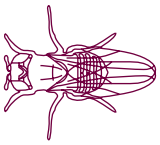
It has long been recognized that simple organisms carry genes that have functional equivalents in humans. Conclusive evidence for this phenomenon was determined when human DNA sequences expressed in yeast cells rescued the phenotypic defects arising from mutations in yeast or *Drosophila* genes, allowing the homologous human DNA sequence to be cloned.[2] Conservation of a given gene through evolution suggests that its protein product performs essential functions

SACCHAROMYCES CEREVISIAE

~7000 genes
~12 million bases

Key features

- Single cell
- Viable as haploid
- Phenotypic variation during cell cycle
- Use of selectable markers
- Ease of plasmid insertion and homologous recombination
- Rapid replication and large cell number
- Few genes and little intronic DNA
- Sequenced genome



DROSOPHILA MELANOGASTER

~15,000 genes
~132 million bases

Key features

- Multicellular but discernible at the single-cell level
- Rapid development
- Sequenced genome
- Short life cycle
- "Tumor" development
- Easy assessment of noncell autonomous function
- Polytene chromosomes
- Large living libraries of mutants
- Sequenced genome

DANIO RERIO

~20,000 genes
~1547 million bases

Key features

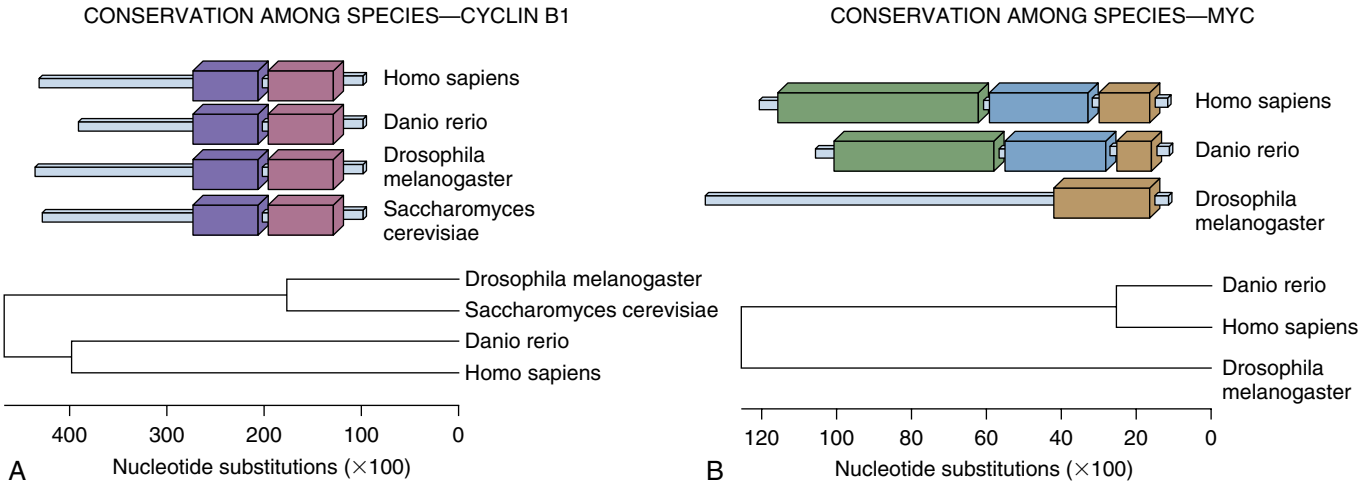
- Vertebrate
- Transparent embryos
- Large numbers of progeny
- Rapid ex-utero development
- Develop true human-like cancers
- Morpholino technology for transient gene knockdown
- Genome editing for targeted gene deletion
- Lines can be maintained as frozen sperm
- Transgenesis is well developed
- Sequenced genome

Figure 8-1 Model organisms (the budding yeast—*Saccharomyces cerevisiae*; the fruit fly—*Drosophila melanogaster;* and the zebrafish—*Danio rerio*) and their key attributes as models for dissecting the molecular basis of cancer.

that are similar across species. The more highly conserved the gene, the more important or critical the function of its product. For example, the amino acid sequence of the fission yeast ribosomal protein of the small subunit 14(rpS14) is 75% identical to the human RPS14. This suggests that from the millions of random mutations arising over millions of years of evolution, only 75% diversification of the amino acid sequence occurred, presumably because the majority of mutations resulted in deleterious effects on the fitness of the organism. Similarly, the most critical functional domains of a protein can be implied by determining the regions that are most highly conserved among species (illustrated in Figure 8-2). The genetic complexity of higher eukaryotes, however, indicates that not all genes are conserved. Indeed, even with the now-vast repositories of bioinformatics that house sequence data for hundreds of genes and species, it is not always straightforward to identify a single gene that is the functional ortholog of a human gene in a simple organism. As a result, in simple organisms there are often fewer genes performing a particular function than in humans. An example of this is the number of functionally conserved BCL2 family members in the intrinsic apoptosis pathway seen in worms and zebrafish (with none identified in yeasts).[3-5] This is further complicated in zebrafish, where whole-genome duplication that occurred in teleost fish more than 150 million years ago[6] has resulted in the presence of two or three copies (paralogs) of a large number of individual genes.[6] Several methods permit determination of which copy of such genes is functionally homologous to the human gene. The analysis of genetic synteny using bioinformatic approaches has demonstrated that zebrafish orthologs of human genes can usually be found in chromosomal locations in the fish that reflect their location on conserved human chromosomal regions.[7] In many cases divergence in promoter sequences leads to tissue-specific activities of different teleost orthologs of a single mammalian gene (see Figure 8-2, C).

Forward Genetics, Reverse Genetics, and Transgenesis

Simple model organisms, with their rapid development and large numbers, lend themselves well to the identification of new oncogenes, tumor suppressors, and novel therapeutic targets important in cancer. This is generally accomplished using classical (and variations on classical) genetic screens. Forward genetic screens are based on Mendelian inheritance of genes and the observation of a phenotype in cells or organisms where a gene has been disrupted (Figure 8-3). The methods by which genes are disrupted and the assays used to screen for phenotypes are wide ranging, and some are species specific. Early screens looked for naturally occurring phenotypes of simple processes, such as inability of yeast to grow at a certain temperature, as a method of assaying genes involved in cell division. To increase the number of mutated organisms (mutants) with a phenotype of interest, methods of inducing mutations in genes, either chemically with retroviruses or with transposons ("jumping genes"), have been employed. Large numbers of organisms



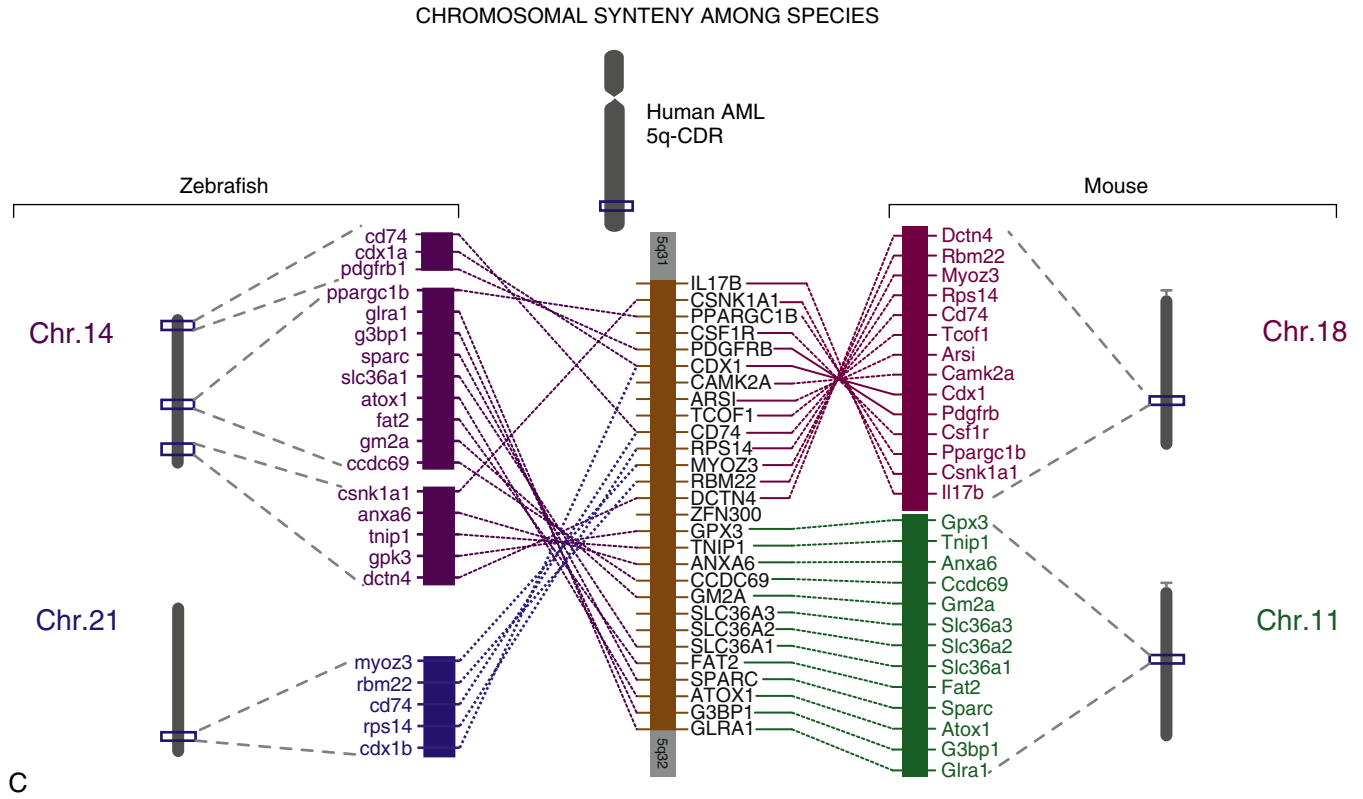


Figure 8-2 Conservation among species for (A) cyclin B1 and (B) the Myc oncogene. Each colored block represents a conserved protein domain. The phylogenetic trees below demonstrate common ancestries and the approximate number of nucleotide substitutions that have occurred leading to the divergence of the proteins for each of the given species. **(C)** The syntenic relationships among zebrafish chromosome 14 and 21, mouse chromosomes 18 and 11, and the region on the long arm of human chromosome 5 that is found to be critically deleted in myelodysplastic syndromes and acute myeloid leukemia with isolated loss of chromosome 5. *AML*, Acute myeloid leukemia; *CDR*, critically deleted region.

are screened to ensure that every gene within the genome has been mutated at least once—this is known as saturation of the genome. Forward genetic approaches allow unbiased isolation of phenotypically mutant organisms, after which the gene causing the mutant phenotype can be identified. The forward genetic screen remains the most powerful strength of model organisms as a cancer model, in particular for the discovery of novel tumor suppressors. Tumor suppressors are genes whose normal functions are to regulate uncontrolled or abnormal growth, or the growth of cells whose genetic integrity has been compromised. Because the cancer-causing effects of these genes can only be fully recognized when they are absent or nonfunctional, forward screening of large numbers of simple organisms is an attractive means to identify such genes in vivo. Historically, a potential drawback of such forward screens, particularly in

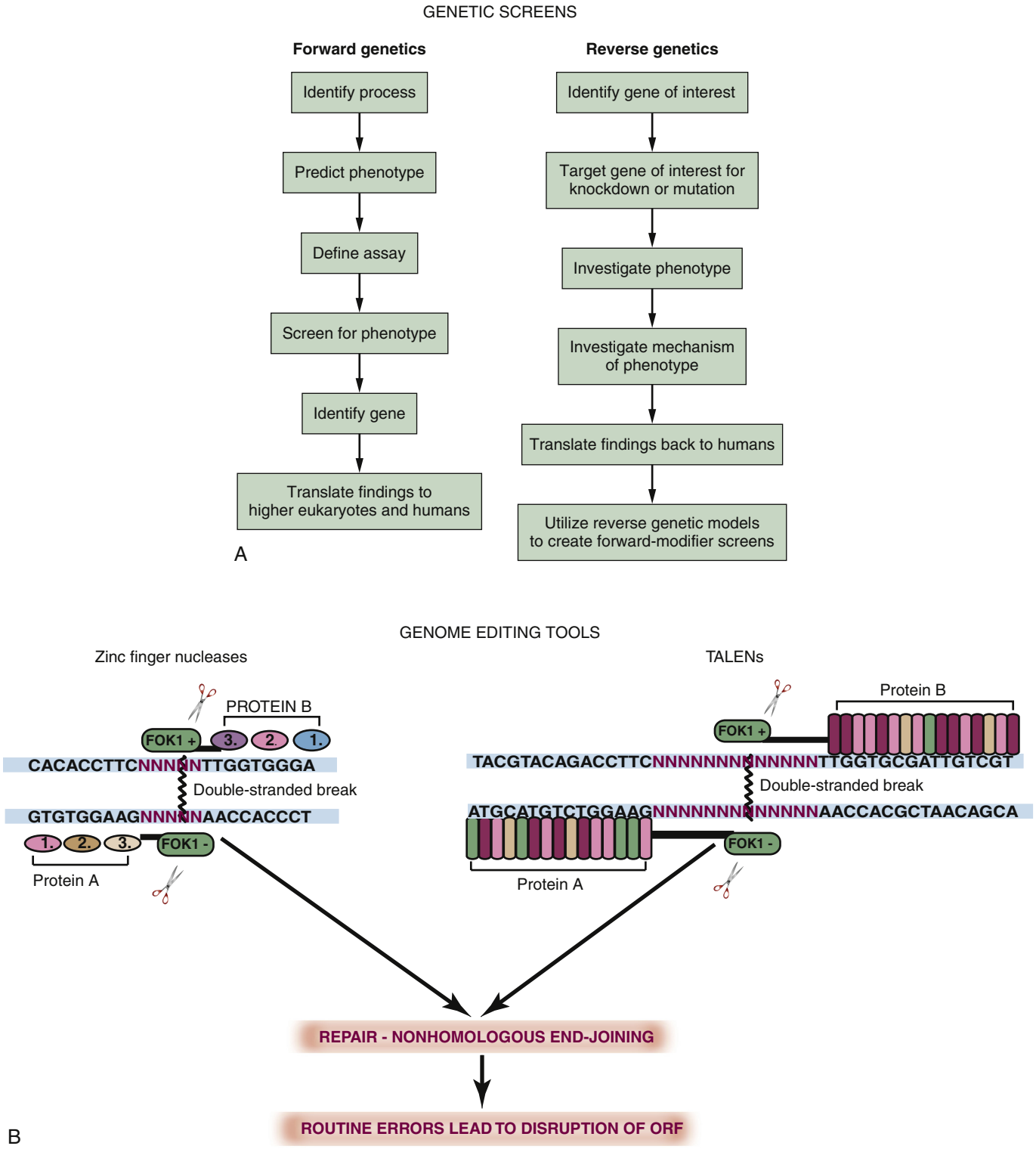


Figure 8-3 **(A)** Flow diagram illustrating the steps involved in forward and reverse genetic screens. **(B)** Schematic of the mechanism by which zinc finger *(left)* and tal effector endonucleases *(right)* function as genome editing tools. Zinc fingers are composed of two half-sites with three separate zinc finger domains in each. Each triplet is fused to the cleavage portion of the chimeric endonuclease FokI. Binding of the zinc fingers to the DNA target site results in FokI alignment for dimerization and cleavage of double-stranded DNA. Tal effector nucleases function in a similar manner. Each half-site is made of 14-16 tal effector repeats. Depending on two critical residues, these repeats will bind an A, C, T, or G, allowing a high level of specificity of DNA binding and subsequent ligation. Repair of the double-stranded break by nonhomologous end joining results in the introduction of insertions, deletions, or substitutions that result in mutant protein generation. *ORF,* Open reading frame; TALENs, transcription activator–like effector nucleases.

the zebrafish, where there is somewhat greater genetic complexity, is that identification of the genetic mutation causing the phenotype can sometimes be time consuming and technically challenging: something of a "needle in a haystack" search. This is especially true if the mutation occurs in a regulatory or noncoding region of the genome or in telomeric regions of chromosomes where increased amounts of crossing over during meiosis further hamper conventional positional cloning techniques. However, the past decade has seen the emergence of massive parallel sequencing technologies that are now being used to facilitate rapid fine mapping of genetic mutants derived from phenotypic screens.[8,9] These technologies allow hundreds of genes to be sequenced simultaneously, covering the entire genome in such a way that regions of the genome linked to the mutant phenotype can be identified when the fish have been crossed to a strain of a different genetic background. The rapidly decreasing costs and increasing efficiency and availability of these technologies will likely fuel a new wave of studies in coming years, accelerated by advances in deep sequencing for positional cloning.

In addition to forward genetic modeling, it is also possible to disrupt specific known genes (such as known oncogenes or tumor suppressors) to investigate the phenotype produced. This process is termed *reverse genetics*. The most common reverse genetic studies are carried out using transient knockdown techniques. These include RNA interference (RNAi) in flies and antisense oligonucleotides ("morpholinos") in frogs and fish. These techniques are particularly useful in determining epistatic relationships among genes giving rise to a particular phenotype, as several genes can be knocked down simultaneously. More recently, there have been a number of advances in the field of genome editing technologies that have made the generation of stable knockout lines at virtually any site within the genome accessible to researchers using simple organisms.[10,11] This is beneficial because it allows specific domains within a protein to be targeted for mutation and allows propagation of mutations through the germline for studies in later stage juveniles or adult organisms. This is achieved by the creation of synthetic restriction endonucleases that fuse the cleavage portion of FokI, a naturally occurring bacterial restriction endonuclease, to a site-specific DNA binding domain generated using synthetic zinc fingers (ZFNs) or transcription activator–like effector nuclease (TALEN) repeats. Two DNA sequences are targeted, one on either side of the region of the genome where a mutation is desired (see Figure 8-3, *B*). Because FokI requires dimerization to induce cleavage at its target site, this produces site specificity and minimizes off-target mutations. Novel technologies allowing the generation of large numbers of TALENs are likely to make the use of this form of genome editing widespread not only in simple organisms but also in mammalian models and cell culture systems.

Forward and reverse genetic models may be used in combination. Reverse genetics in simple organisms may add tools for the investigation of the genetic interactions of a known gene by permitting the development of modifier screens where the mutant is subjected to forward genetic screening to identify genes that enhance or suppress its phenotype. In this way modifier screens may provide novel therapeutic targets in human cancers.

Another genetic tool used in simple model organisms is transgenesis. To obtain a transgenic organism, specific DNA sequences are typically introduced into the genome of an organism and expressed under the control of a specific promoter sequence to guide the cellular, temporal, and spatial localization of transgene expression. For example, in zebrafish, the introduction of the mouse *Myc* gene under the control of the *rag2* lymphocyte-specific promoter leads to expression of *Myc* in the thymus and the subsequent development of T-cell leukemia/lymphoma.[12,13] Adding the coding sequence of green fluorescent protein to the integrated construct has the additional advantage of permitting spatiotemporal visualization of the development of cancer in this system. A prime example is the recent use of dopamine β-hydroxylase promoter to drive MYCN and activated ALK expression in transgenic zebrafish to produce neuroblastoma,[14] demonstrating the power of this model for detailed studies to reveal cellular mechanisms underlying synergy between oncogenes that are also activated together in human malignancies. Transgenic approaches are easily performed in yeasts, which undergo efficient homologous recombination into their chromosomes, allowing site-specific integration of the transgene. Using this tool in yeasts facilitates reverse genetics by replacing the normal functioning gene with a mutated or nonfunctional form (which may be genetically engineered or even derived from a different species).

Drug Screens

Simple organisms can also be used for drug discovery in a variety of ways. Yeast can be manipulated to express a gene or protein of interest at a higher level than normal, or a heterologous human gene under control of a yeast-specific promoter. A differential effect of a drug on the normal-versus-mutated gene can also be assessed (e.g., attempting to find drug targets selective for certain oncogenes).[15] More recently, drug screens using whole organisms, including both flies and zebrafish, have been successful in uncovering potential novel therapeutic anticancer drugs and for revealing previously unsuspected anticancer activities of existing drugs.[16,17]

Conditional Models

Only a small number of human cancers can be attributed to the presence of an underlying inherited cancer predisposition mutation; thus the majority of human cancers arises from a series of somatic mutations acquired over time. Because many human oncogenes and tumor suppressors play a critical role in embryologic development, germline mutations affecting key genes of this type in all tissues frequently lead to death during development. Although investigation of the embryologic phenotype in model organisms continues to provide crucial information on gene function, the study of genetic interactions in specific tissues and in specific cancer models provides additional information on how the mutated gene contributes to tumorigenesis in vivo.

Over the past 20 years, genetic tools have been developed to engineer targeted conditional expression or (in some cases) knockout of a specific gene. There are three main conditional systems that facilitate directing gene expression to a specific cell type at a specific time. The first is based on an enzyme from P1, which is a bacteriophage that infects the bacterium *Escherichia coli*. This virus produces an enzyme called *Cre recombinase* that cuts DNA whenever it sees two identical 34-base-pair sequences known as Lox-P sites. The enzyme removes the DNA between the two Lox-P sites, and the sites are ligated together. In model organisms, this system can be used by driving the expression of Cre with a promoter that is only expressed at a certain site (tissue specific) or with a promoter that is activated by exposure to a specific stimulus such as heat shock or a particular drug (e.g., estrogen). Lox-P sites can be introduced into a transgene such that on Cre activation, the transgene is expressed (or removed) in a specific tissue. Fluorescent proteins can also be incorporated into transgenes to allow visualization of where the transgene is expressed and where the Lox-P sites have been removed (Figure 8-4). In a similar fashion, Flp recombinase is an enzyme made by the 2-μm plasmid of *Saccharomyces cerevisiae*. This recombinase acts in a similar way to Cre recombinase, recognizing two 34-base-pair sequences termed *Frt sites*. This system has been extensively employed in flies, where instead of simply excising the intervening DNA sequence between two Frt sites, Flp recombinase results in the crossing over and exchange of genetic material between arms when Frt sites are located on opposite arms of the same chromosome. This allows, for example, the expression of mutant tissue in an otherwise wild-type background[18] (see Figure 8-4). Tissue-specific overexpression of a gene can also be achieved by using the yeast transcription factor Gal4 driven by a tissue-specific promoter and its upstream activating sequence (UAS) driving the gene of interest. UAS can also drive a fluorescent protein–colored marker to allow spatial localization of cells expressing the gene of interest (see Figure 8-4).

Yeast

Genetic Tools and Functional Genomics

Recovery of the genes responsible for a mutant phenotype observed in a forward genetic screen and harnessing the power of reverse genetics is made possible in yeast by the ease with which the organism can be transformed with DNA using plasmid vectors or polymerase chain reaction (PCR) products and by the subsequent ability of the yeast to undergo the process of homologous recombination. Homologous recombination is a conserved DNA repair process that allows recognition of homologous DNA sequences at sites of double-stranded DNA breakage. This recognition allows double-stranded breaks to be repaired by using the other intact copy (located on the sister chromosome) as a template. This physiological mechanism is hijacked when vector-derived DNA is designed to carry homology arms with sequences identical to the genome location where incorporation is desired. The yeast homologous recombination machinery then recognizes the homology arms and inserts the vector DNA sequence (chosen by the researcher) into the desired location. In this way, any gene can be replaced by another stretch of DNA. This could be a mutated or null allele or a normal allele to replace a mutated one. In addition, mutant or wild-type alleles can be coupled with genes encoding positive or negative selection factors to allow the identification of yeast strains that have undergone homologous recombination (such as an antibiotic or growth factor requirement). The application of these techniques is widespread, including the development of targeted gene "knockouts" such as those used in the yeast genome deletion project. An example of this is shown in Figure 8-5. Molecular profiling of the effects of different growth conditions has been facilitated by the addition in the yeast genome deletion project of two unique 20-nucleotide tags that flank each knockout cassette. Using these tags as "barcodes" and starting with equal quantities of each deletion strain, gene chips have been developed to hybridize and quantify each tag (and thus knockout strain) in any given growth condition.[19]

Because the yeast genome is relatively small and contains little noncoding DNA, it has been possible to derive plasmid libraries for all genes found in yeast that are of a manageable size. This is done by cutting the whole genome with restriction enzymes that recognize a particular DNA sequence and then inserting each of these smaller cut pieces of DNA into a plasmid vector. These DNA libraries are useful to identify the gene causing a phenotype or to address the phenotype resulting from the overexpression of certain genes (e.g., which can also be used in a drug screen).

One complication of examining genotype-phenotype relationships is that for some genes loss of function and/or

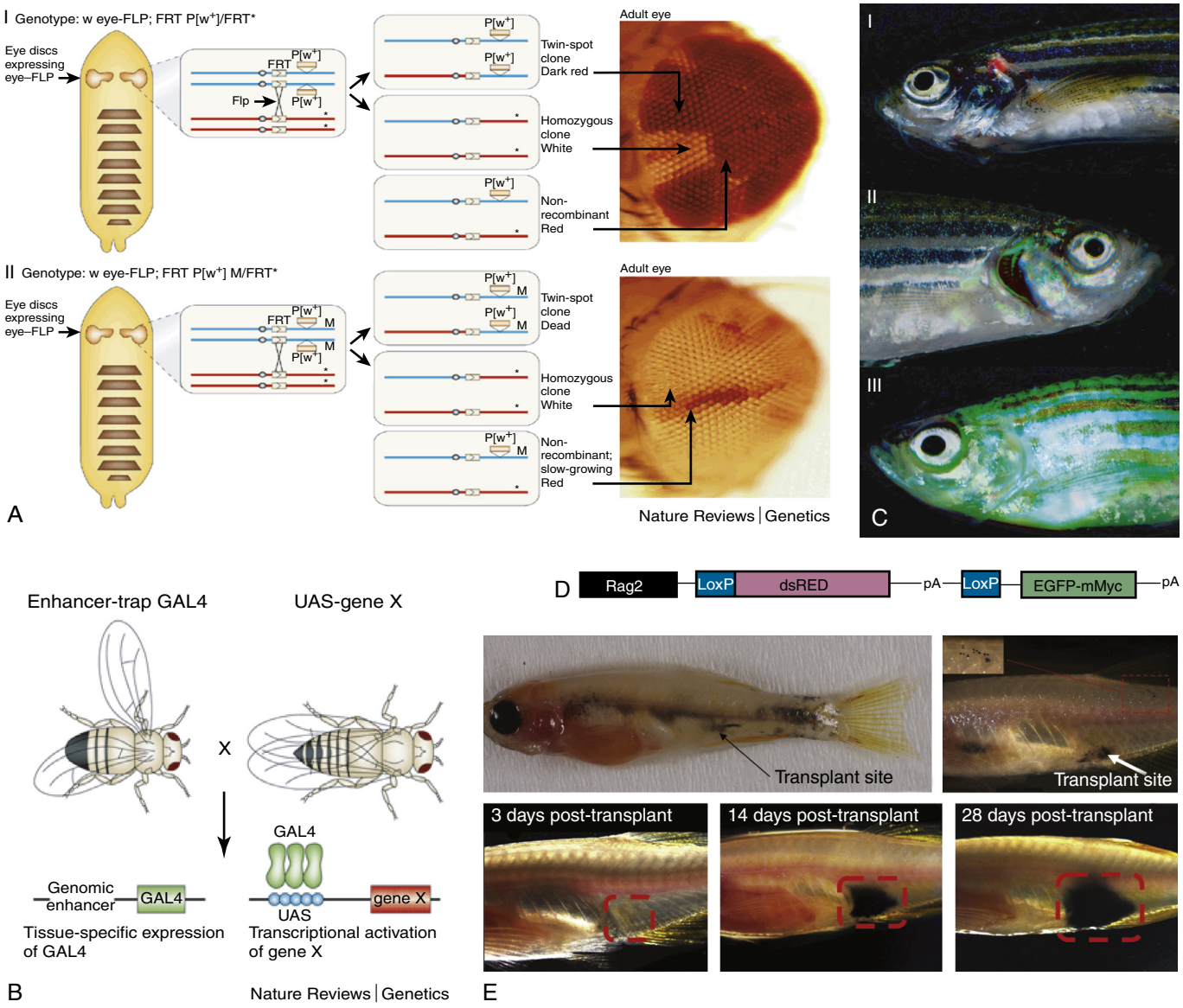


Figure 8-4 **(A)** Flt/FRP system/twin-spot clones. By placing the FLP recombinase gene under the control of the eyeless enhancer (which drives expression specifically in the eye-antennal imaginal disc), Flp/FRT-mediated recombination can be targeted to this disc to generate homozygous mutant clones in the eye in flies that are otherwise heterozygous. *(I)* The non-mutant chromosome (the asterisk indicates a mutation) is marked by a mini-white transgene, but there is no selection against the twin-spot clones or nonrecombinant cells, and both the mutant clones *(white)* and the twin-spot clones (*darker red*, because they carry two copies of white+) are relatively small. *(II)* The effects of incorporating a Minute mutation (M) onto the nonmutant FRT chromosome. The mutant clones now occupy almost all of the eye, because they outcompete the slow-growing nonrecombinant cells (which are M/+), whereas the twin-spot clones die. **(B)** Gal4/UAS system. The yeast transcriptional activator Gal4 can be used to regulate gene expression in *Drosophila* by inserting the upstream activating sequence *(UAS)* to which it binds next to a gene of interest (gene *X*). The *GAL4* gene has been inserted at random positions in the *Drosophila* genome to generate "enhancer-trap" lines that express *GAL4* under the control of nearby genomic enhancers, and there is now a large collection of lines that express *GAL4* in a huge variety of cell-type and tissue-specific patterns. Therefore, the expression of gene *X* can be driven in any of these patterns by crossing the appropriate *GAL4* enhancer-trap line to flies that carry the *UAS*–gene *X* transgene. This system has been adapted to carry out genetic screens for genes that give phenotypes when misexpressed in a particular tissue (modular misexpression screens). **(C,D)** Cre-Lox system. Zebrafish carrying the construct shown in **(D)**: *(I)* No cre activation—fish show red thymus, showing that the dsRED cassette has not been excised. *(II)* Following cre injection, the dsred stop cassette is removed, allowing expression of the cMyc-EGFP fusion oncogene. This fish has an enlarged green thymus indicating the development of T-cell acute lymphoblastic lymphoma. *(III)* In this older fish, the whole fish fluoresces green, indicating disseminated T-cell acute lymphoblastic leukemia. **(E)** Tumor transplantation experiment using the transparent "casper" fish. These images show the development of melanoma (derived from p53 null/BRAFV600E model) over time in the same animal. The tumor can be monitored externally without sacrificing the fish. *(**A** and **B**, Reprinted with permission from Macmillan Publishers Ltd:* Nat Rev Genet. *St. Johnston. **C**, Courtesy Hui Feng (unpublished data). **E**, Reprinted from White et al.,* Cell Stem Cell. *Copyright 2008, with permission from Elsevier.)*

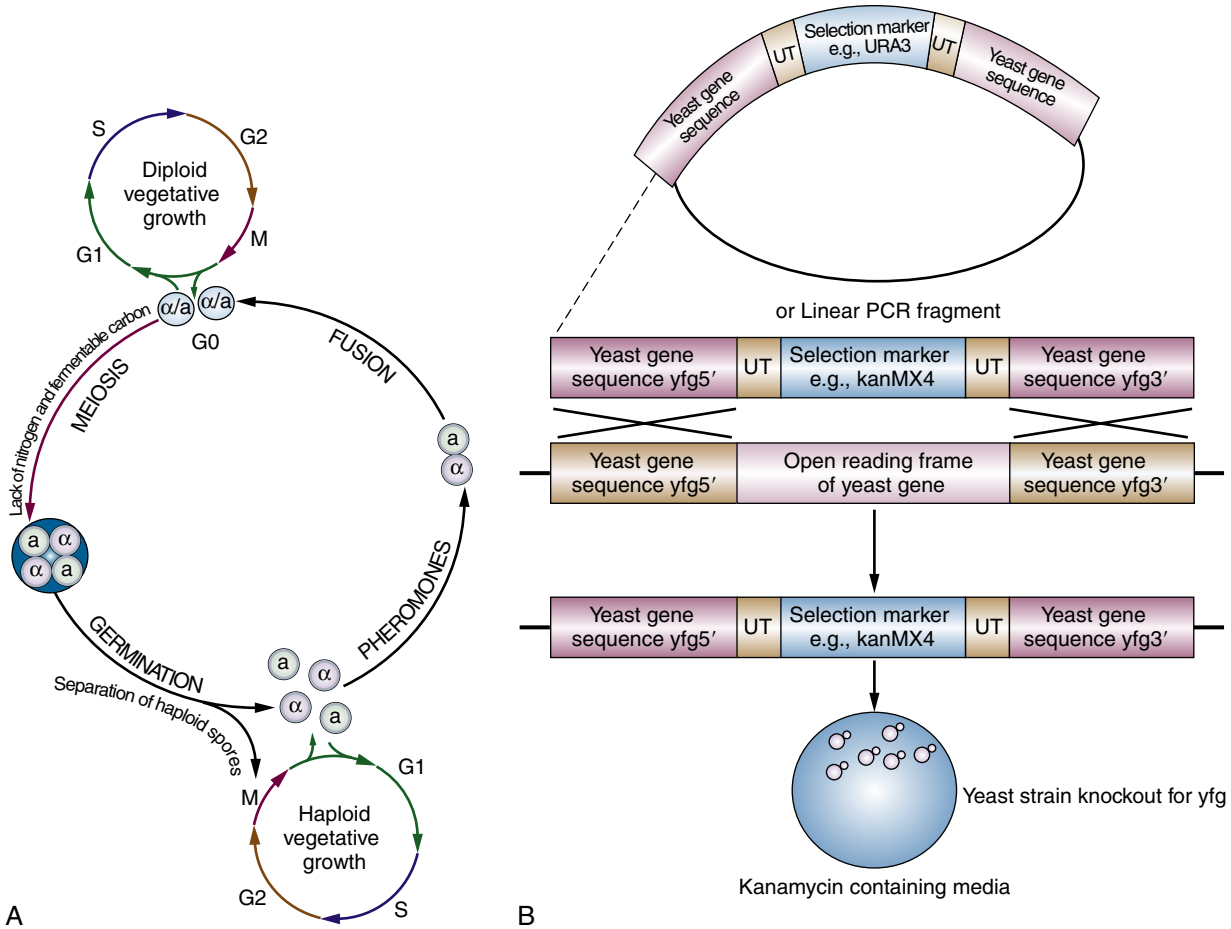


Figure 8-5 **(A)** A schematic representation of the budding yeast life cycle. Under adequate nutritional conditions diploid yeast cells undergo vegetative growth by mitosis *(top left)*. The cells cycle through G1, S (synthesis), G2, and M (mitosis) phases. When starved of nitrogen and fermentable carbon, sporulation occurs with formation of gametes by meiosis. The gametes are contained within a casing called an *ascus*. As the gametes germinate, the haploid spores separate. The a and α gametes secrete pheromones leading to fusion of an a to α gamete when they meet. If separated, however, haploid vegetative growth can also continue by mitosis *(bottom right)*. **(B)** Schematic representation of replacement of yeast gene *yfg* (your favorite gene) with a deletion cassette containing the selection marker KanMX4 conferring resistance to the antibiotic G418 (kanamycin). 40-50 bp of yfg gene sequence at the 5′ and 3′ ends are cloned with KanMX4 selection marker between, flanked on each side by 20-bp unique tags *(UT)*. The *yfg* sequences are recognized by the homologous recombination machinery in the yeast, and a proportion of yeasts will swap the genetic material encoding the gene for that encoding the selection marker. Yeasts that survive in media containing kanamycin have incorporated the deletion cassette and no longer contain a functional *yfg*. These strains can be isolated from mixed cultures by PCR using the flanking UT. *(Adapted by permission from Macmillan Publishers Ltd:* Nat Rev Genet. *Kumar A and Snyder M. Emerging technologies in yeast genomics. Copyright 2001.)*

gain of function do not show a phenotype. The likelihood is that these genes are either redundant or act in pathways whose physiological functions are buffered by other cellular pathways acting in parallel.[20] To decipher the interaction between two deleted genes (synthetic fitness or, more severely, synthetic lethality when the interaction causes lethality) or between a deleted gene and an overexpressed gene (known as synthetic dosage fitness or lethality), novel synthetic genetic array (SGA) screens have been employed.[21,22] Yeasts with the mutation of interest in the haploid state are mated to a pool of haploid yeast knockout strains of the opposite mating type. Selection markers are introduced to allow identification of double mutant strains, and their phenotype is assessed. Synthetic lethality screens have been further developed to incorporate the microarray technology using barcodes as described earlier—the synthetic lethality analyzed by microarray (SLAM).[20] SLAM has allowed analysis of double-mutant heterozygous diploid strains of yeast, as well as other genetic interactions such as chemical genetic interactions.[23]

Yeast screens have been devised to determine not just genetic but also protein-protein interactions. These can be in the form of two-hybrid screens, or more recently in the form of tandem affinity purification tagged (TAP-tag) analysis. The principle of the two-hybrid screen analysis is that transcription factors require two domains—a DNA binding domain (BD) and an activation domain (AD) in close proximity to one another—in order to bind to and cause transcription of their target gene. Part of the Gal4 transcription factor (either the BD or the AD; for this example the BD) is fused to the protein of interest, for example, the MYC oncoprotein. A library of other proteins is then fused

to the Gal4 AD. If any of the library proteins interacts with MYC, then the Gal4 transcription factor will bind and cause transcription of target genes. The target gene can be engineered to be a selection factor that will only allow yeast to grow in the presence of its expression in selective culture conditions, and its identity can be confirmed by subsequent sequencing.[24] In TAP-tag analysis, a tandem affinity purification protein tag is introduced into the open reading frame (ORF) of each yeast gene to create the expression of a fusion protein. This protein tag is specially designed to allow efficient affinity purification of the tagged protein of interest along with other proteins bound or associated with it. The resultant interacting proteins can then be resolved by a variety of methods, the most accurate of which is mass spectrometry.[25,26]

The Cell Cycle

The fundamental processes of cell growth and division are governed by the tightly regulated processes that maintain the cell cycle. The cell cycle is an ordered set of events by which cells grow and divide to produce two identical daughter cells. It is divided into four phases, as shown in Figure 8-5. Two features of cancer cells in mammalian systems are controlled (at least in part) by cell cycle maintenance: altered growth and genomic instability. Based on a number of features, yeasts provide the ideal system for study of the cell cycle.[27] The budding yeast *Saccharomyces cerevisiae* has been the most highly investigated. *S. cerevisiae* is able to survive in both haploid and diploid states. In the presence of sufficient cell nutrients, diploid cells undergo division by mitosis and growth by budding. The size (or presence) of a bud can be visualized by light microscopy and permits determination of cell cycle status in individual cells. Each diploid cell contains a copy of each of the two mating types—Mat**a** and Matα—determined by the MAT locus. When diploid cells are starved of nitrogen and fermentable carbon, they undergo sporulation and commence formation of **a** and α gametes. Here the cells undergo division by meiosis, followed by differentiation of the subsequent haploid progeny. The haploid progeny immediately fuse with a cell of the opposite mating type to produce a diploid cell—a process determined by a mating pheromone that is specific to each mating type (**a** or α). Therefore the cell cycle and mating machinery are integrally linked.[28,29] In addition, yeast cells that have arrested at different parts of the cell cycle (usually under specific conditions such as high temperature) have disrupted genes critical to progression through the cell cycle and cell growth, which may be potential oncogenes and/or tumor suppressors. The study of mutant yeast strains with abnormalities in the cell cycle has led to the identification of genes orthologous to human oncogenes and tumor suppressors.

CDC2 and CDC28

In 2001, the power of yeast as a genetic tool for studying the cell cycle and its implications in cancer were recognized with the award of the Nobel Prize in medicine to Paul Nurse and Leland Hartwell. They not only elucidated many of the critical genes involved in regulating the cell cycle but also demonstrated the genetic and biochemical conservation that makes study in this and other simple organisms so powerful. Two of the critical genes identified by these investigators continue today to provide insight into how cancer cells evade normal growth and cell cycle regulatory mechanisms. The *CDC28* gene was discovered in the early 1970s by Leland Hartwell in an early genetic screen to identify *S. cerevisiae* strains that conditionally blocked cells at different stages of the cell division cycle (cdc). The presence of Cdc28 was found to be essential for cells to initiate both the nuclear and cytoplasmic events required for cell division. Before the Cdc28-dependent step, yeast cells are able to undergo either sexual replication or entry into the cell cycle, and thus the Cdc28 step in G1 came to be known as the start of the cell cycle in the budding yeast. The *CDC28* gene was cloned in 1980, and by 1985 Cdc28 was shown to have protein kinase activity.[30]

Paul Nurse uncovered a critical regulator of the cell cycle in *S. pombe*. Using the observation that in budding yeast, cell size is coordinated with progression through the cell cycle, a number of "wee" (meaning small) mutants were identified. "Wee" mutants progressed through the cell cycle more rapidly than normal before sufficient time for normal growth had passed, resulting in the generation of the small phenotype. The second "wee" mutant isolated, *wee2*, was shown to map the same locus as *cdc2* and later, in 1982, was shown to be functionally homologous to the *CDC28* gene in budding yeast. The cloning of the human *CDC2* gene in 1987 confirmed the utility of this system for investigating the role of specific genes in humans.[27] In more recent years, *cdc2* and *CDC28* have been renamed *CDK1* (cyclin dependent kinase 1) as part of the CDK family of kinases, which (along with other roles in cell cycle, transcription, and differentiation) associate with cyclins, allowing intricate control of the cell cycle. CDK1 associates in particular with cyclin B and is involved in the control of mitosis (reviewed in Refs. 31 and 32). Since then, abnormal expression of CDK1 has been found in a variety of human cancers and is now known to be required for efficient phosphorylation of the Bloom syndrome DNA helicase (BLM).[33] Homozygous mutation of the BLM leads to Bloom syndrome—a condition in humans

that predisposes to multiple forms of cancer as a result of genomic instability.

Ploidy, Genome Instability, and Cancer

The study of Leland Hartwell's temperature-sensitive cell division mutants led to the analysis of genes essential for cell cycle progression and their roles in determining the fidelity of genetic replication. Several cell cycle mutants demonstrated markedly increased rates of chromosome loss, recombination, or mutation. From this evidence, the DNA damage checkpoints and DNA repair pathways active during the cell cycle were first identified. One such mutant carried a mutation in *POL3*, a gene now known to encode the catalytic subunit of DNA polymerase subunit δ. This polymerase forms part of a proofreading enzyme whose role is to limit errors occurring during DNA replication by removing any incorrectly incorporated nucleotides via intrinsic exonuclease activity. Homozygous mutations in *POL3* are embryonic lethal (in yeasts and mammals), but heterozygous mutations within the catalytic domain result in a mutator phenotype. The mutator phenotype—where a mutation in one gene predisposes to the development of additional mutations—is known to contribute to the development of cancer in mammals, and thus heterozygous mutations in *Pol3* result in increased rates of cancer in mice. However, in bacteria it is known that the presence of a mutator phenotype results in reduced fitness of the bacterium. This occurs because with each division, an increase in the number of deleterious mutations occurs, ultimately reducing its ability to grow and divide. This continues until such time as an "antimutator" mutation occurs that decreases the number of de novo mutations, allowing this subpopulation to have the growth advantage. It is possible that such a process also occurs in humans, contributing to the clonal evolution of tumors over time. Herr et al. recently tested whether this phenomenon was active in eukaryotic cells using budding yeast, with the hypothesis that a critical threshold of mutations could be tolerated before a yeast became incapable of further cell division.[34] The authors identified several mutations within conserved residues of *Pol δ* that exacerbated the mutator phenotype observed in a yeast strain also carrying a mutation within the mismatch repair gene *MSH6 (msh6Δ)*. In addition to confirming their hypothesis that a threshold number of mutations can be tolerated within a eukaryotic cell, the authors identified occasional yeast colonies that grew in the presence of apparently otherwise synthetic lethal gene mutation combinations. Assessing these "error-induced extinction" (eex) mutants, they determined that indeed these yeasts had developed antimutator phenotypes, with a third of cases having additional antimutator mutations within the *POL3* gene itself as the mechanism of their escape. This elegant study highlights evidence that supports some recent clinical observations in cancer, such as the remarkable success of inhibition of the poly(ADP) ribose enzymes (PARP). PARP1 and PARP2 enzymes are an integral component of the repair of single-stranded breaks as cancer therapies. In the presence of mutations in genes involved in homologous recombination, such as BRCA1 and BRCA2, found in breast cancers among others, PARP inhibition results in selective tumor killing.[35] The likely mechanism of this is the synthetic lethality of tumor cells that arises from the breaching of the critical threshold of mutations sustainable by an individual cell. However, it does raise the concern that the clonal evolution of cells resistant to this mechanism of killing is likely to be problematic when using this therapeutic strategy in some cases.

Flies

Why Use a Fly?

Drosophila melanogaster has been used as a model organism in developmental biology and genetics for a century, and the resultant generation of a vast array of tools make it one of the most comprehensively genetically tractable systems for study. Several features of the fruit fly have led to its popularity as a genetic model, most critically the small number of genes contained within the four fly chromosomes, relatively little redundant DNA, and large numbers of human homologs that have been identified following the complete sequencing of both the fly and the human genomes. In addition, fruit flies develop large polytene chromosomes in the salivary gland. These chromosomes are produced in the last larval stage (the third larval instar) when large amounts of glue proteins are required for pupation. The large amount of protein production is achieved by genome amplification by a process called *endoreduplication*—DNA replication without division. When stained by standard G-banding, the resolution of endoreduplicated chromosomes is an order of magnitude greater than that seen of human chromosomes, as there are multiple copies of each gene. This in turn facilitates the identification of genes that have been deleted. Although wild-type flies do develop tumors, the similarities of these spontaneous growths to mammalian cancers are limited. However, forward genetic analysis has uncovered cancer-like proliferations in the developing fly larva that provide an excellent platform for the investigation of tumorigenesis in this multicellular organism. Early in embryogenesis, cell fate is assigned, leading to the formation of certain adult structures, which develop in the larva via saclike invaginations of specialized epithelium (known as imaginal discs). There are 15 imaginal discs—seven bilaterally symmetrical pairs and

one germ cell imaginal disc. Imaginal discs consist of a single layer of cells that can be easily visualized in the developing larva. In addition, both brain and blood cell neoplasia can be seen in mutant fruit flies.[36] Not all aspects of human cancer can be modeled in a fly, however. In particular, flies lack a closed vascular system, and thus angiogenic properties of tumors cannot be investigated. Despite this, genes known to regulate the angiogenic properties of human tumors, such as vascular endothelial growth factor (VEGF), have fly homologs that have been implicated in tumor development in flies.

Another feature of the fruit fly that makes it an attractive model to study is the availability of large banks of mutant flies. Mutagenesis in flies has been performed using x-rays, chemical agents to induce point mutations, and P-element–mediated insertional mutagenesis. P-elements are transposons or sequences of DNA that can move around or "jump" within the genome. When transposons insert into the genome at the beginning of a coding sequence, that gene's transcription is disrupted, generally creating a null allele. Identification of the disrupted gene is much easier than with a chemically induced point mutation, because the sequence of the P-element is known and can be "tagged," and PCR primers can be used to facilitate gene identification. To determine which flies carry the P-element, its sequence can also be modified to carry a marker, such as rosy eyes. "Jumping" of the P-element requires the function of another gene, transposase, and thus the disrupted gene will be fixed unless the transposase is present. The transposase gene can be bred into flies carrying a P-element to induce the latter to move and is usually carried on what is known as a "balancer" chromosome with another mutation that is easily identifiable, such as curly wings.

Genetic Tools

The first tumor suppressors identified by forward genetics in flies exhibited only one or two features of cancer. The phenotypes observed were of hyperplasia or neoplasia in a single tissue affecting 100% of flies with the mutations and, unlike in human cancers, there did not appear to be a requirement for the development of additional mutations to cause tumorigenesis. Because these initial tumor suppressor genes were not homologous to those found in humans, the fly became less popular as a model organism to study cancer. However, this soon changed, as highly conserved signaling pathways were subsequently identified in flies and humans and more sophisticated genetic techniques to study gene interactions became available.[37] These included genetic screens to identify second-site modifiers of known tumor suppressors; the discovery of a *Drosophila* homolog of C-terminal src, dcsk, isolated by Stewart et al. in a screen for dominant modifiers of the lats tumor suppressor[38]; and the dissection a number of second-site modifiers of the transcription factor E2F.[39,40]

More recently, the use of the conditional Frt/Flp and Gal4/UAS systems has been invaluable in cancer research in the fly. Focusing on the fly's greatest strength—that it offers the ability to investigate cell-cell interaction at a single-cell level—the introduction of mosaic clone analysis for the first time underpinned the role of the microenvironment and non–cell-autonomous cues in the life of an individual cell.[41] The utility of this tool has been used to dissect the cell-autonomous and non–cell-autonomous roles of the *Drosophila myc* gene.[42-44] Similarly, the interactions between oncogenes and tumor suppressors have been evaluated in mosaic clones, demonstrating cooperation between many known oncogenes and fly tumor suppressors.[45-48]

The normal processes by which cells migrate during different developmental processes have been studied extensively in the fruit fly. Different processes use different modes of migration, requiring alterations in cell polarity, cell shape, and the adhesion of cells both to other cells and to the extracellular matrix. Disruption of genes and signaling pathways employed in normal migration processes has been shown to be involved in the ability of cancers to invade local tissues and metastasize to distant regions. This area of research is in its infancy, but the extensively delineated normal processes will undoubtedly assist in the investigation of mechanisms by which cancer cells evade their local environment in this model.[18]

Numerous ingenious second-site modifier and overexpression screens have also been developed in *Drosophila*, the complexity of which are beyond the scope of this chapter but are extensively reviewed elsewhere.[1,18,49]

Malignant Neoplastic Tumor Suppressors in *Drosophila:* scribble and Others

Many fly mutant genes were classified as tumor suppressors in early embryonic screens for larval tissue overproliferation. However, subsequent characterization of a number of those genes demonstrated mechanisms of tissue expansion that did not represent features normally ascribed to cancer cells, and thus these genes are no longer considered true tumor suppressors. Despite this, the recent identification of the tumor suppressor *scribble* was through a screen designed to identify maternal effect mutations that disrupted aspects of normal epithelial morphology.[45] It was noted that *scribble* mutants, in addition to disrupting epithelial morphology in the embryo, also led to epithelial defects in the monolayer epithelium of the female germ cells (follicle cells), in which clones of mutant cells were expressed among wild-type cells.[45] A further screen for additional mutant clones affecting the follicle

cell epithelium led to the identification of another mutant with a phenotype almost indistinguishable from that of the *scribble* mutants. Mapping of this mutation revealed it to be an allele of a previously identified tumor suppressor called *lethal giant larvae (lgl)*. It was also noted that mutant clones of another known tumor suppressor, *discs large (dlg)*, led again to a very similar phenotype in follicle cells. In normal tissues, the role of the Scribble protein is in maintenance of cell polarity and cell-cell adhesion, by controlling the localization of other proteins within epithelial cells in order to maintain correct spatial orientation. Bilder et al. postulated that given such similarities in phenotype *scribble* may be a tumor suppressor like *lgl* and *dlg* and also that *lgl*, *dlg*, and *scribble* might interact to form the necessary machinery to maintain cell architecture and cell proliferation in fly epithelium.[45] The identity of *scribble* as a tumor suppressor was confirmed by investigation of the epithelium of the third-instar larvae imaginal discs, which demonstrated cellular overproliferation with loss of apicobasal polarity and disordered architecture. In addition, overgrowth of brain tissue was observed in *scribble* mutants—another feature common to the *lgl* and *dlg* mutants. Epistatic relationships among the three genes have also been demonstrated by the ability to enhance the phenotype of *scribble* mutants by an additional heterozygous mutation of either *dlg* or *lgl*. Following the discovery of *scribble* and its properties as a tumor suppressor in flies, a further screen set out to use the fly as a method of studying the metastatic properties of tumors. Until this point, individual tumor suppressors and oncogenes that had been studied in flies apparently lacked the capability to proliferate without the microenvironment in which the malignant cells reside. This is perhaps not surprising, given the premise that a single genetic lesion is rarely sufficient to promote tumorigenesis, but that it creates a mutator phenotype predisposing to the additional mutations required for cancer to develop; and that in an organism such as the fly, the likelihood of that secondary mutation occurring is low within its short natural lifespan. The screen that was developed investigated the interaction of activated *ras* (ras^{v12}) and known and unknown additional mutations. The system allowed for the development of clones of malignant cells that expressed ras plus additional mutations in the normal microenvironment within the eye disc, using the Frt/Flp system, the Gal4/UAS, and the Gal80 suppressor to localize expression. The results demonstrated that the combination of a ras^{v12} mutation and *scribble* mutation led to circulating tumor cells within the fly hemolymph open circulation and the development of widespread metastatic tumor formation. In these metastatic tumors, basement membrane integrity was breached (as in mammalian metastatic tumors), and overexpression of the junctional adhesion protein E-cadherin suppressed the metastatic behavior of the tumors. This is also in keeping with mechanisms of metastasis in human epithelial tumors, where E-cadherin is frequently downregulated.[50] These studies unequivocally demonstrate the utility of the fly as a cancer model with unique properties for uncovering novel genetic interactions and potential therapeutic targets. Recent studies on the human *SCRIBBLE* gene have shown that it is downregulated in cervical cancer[51,52] and the majority of invasive breast cancers[46] and interacts with the adenomatous polyposis coli gene, leading to altered expression in many cases of colon cancer.[53]

Archipelago

The archipelago *(ago)* gene was identified in a screen to distinguish mutant clones in the eyes of fruit flies that provided a proliferative advantage over their wild-type neighbors. The screen identified several known tumor suppressors as well as three alleles of a novel gene the authors named *archipelago*. *ago* mutant clones showed increased proliferation compared to wild type and only a small amount of compensatory apoptosis. *ago* encodes an F-box protein. F-box proteins are involved in the recognition of other proteins, such as *myc* and *cyclin E*, which are targeted for degradation by a series of enzymes that catalyze the addition and polymerization of the small protein ubiquitin. These specificity factors are termed *E3 ubiquitin ligases*. Polyubiquitination directs the protein to the proteosome for degradation. The F-box protein exists in a complex with other enzyme components required for ubiquitin activation (E1) and ubiquitin conjugation (E2). In *ago* mutants, all three alleles were found to be mutated in the domain of the protein known to be involved in substrate recognition (known as the WD repeats). This led to the hypothesis that the mutant *ago* was unable to recruit a protein substrate for degradation, and this in turn was responsible for the observed phenotype. Because of the proliferative phenotype, the authors hypothesized that a positive regulator of the cell cycle may be involved in the observed phenotype and investigated expression levels of the cyclins. Levels of cyclin E protein were found to be increased without a corresponding increase in cyclin E mRNA, suggesting a post-transcriptional mechanism. *Cyclin E* complexes with *Cdk2* (cyclin-dependent kinase 2), and degradation of this complex promotes the transition from the G1 to S phase of the cell cycle. In the presence of excess cyclin E, cells are driven to replicate their DNA prematurely, leading to genomic instability. Therefore Ago appears to be the F-box protein that directs ubiquitination and subsequent degradation of cyclin E, and the failure to degrade cyclin E is responsible for the proliferative phenotype observed. Elevated cyclin E levels are seen in a variety of human cancers, including breast and ovarian cancer. The human *AGO* ortholog *FBW7* (also known as

hAGO, *hCDC4*, and *FBXW7*) was shown to be mutated in 4 cancer cell lines, including 3 of 10 ovarian cancer cell lines and 1 T-ALL cell line.[54] A further report confirmed the role of human *FBW7* and *Drosophila ago* as part of a complex of proteins responsible for E3 ubiquitination known as an *SCF complex* and showed reduced levels of FBW7 mRNA in breast cancer cell lines where cyclin E levels were elevated.[55] Subsequent investigation has shown that a small number of primary ovarian cancers have mutations in *FBW7*.

Fish

In 1995, Christine Nusslein-Volhard won the Nobel Prize in medicine for her work on the delineation of the embryonic axes of the developing fruit fly. Notably, half of her acceptance speech was dedicated to a different organism, the zebrafish.[56] The major appeal of the zebrafish over other organisms as a cancer model is that it is allows investigation of vertebrate tumor biology but remains amenable to embryonic and forward genetic study in a manner quite unfeasible in other vertebrates. The transparent zebrafish embryos undergo extrauterine fertilization and development. The embryos can be maintained in the haploid state, and gynogenetic diploid (diploid fish derived from maternal sister chromatids—or half-tetrads) are viable to adulthood and fertile. Each female fish is capable of producing up to 200 eggs per clutch, and in vitro fertilization from frozen sperm is also possible. Embryonic development is rapid, with the completion of somitogenesis in only a few days, and adult fish are able to reproduce from 3 months of age. Although the speed of forward genetic screening is slower than in flies or yeast, zebrafish is the model of choice for large-scale forward genetics in a vertebrate organism.[57,58] The addition of a beating heart and closed circulation in zebrafish provides the ability to dissect additional facets of cancer biology, such as abnormal angiogenesis. In contrast to flies and yeast, fish get cancer in the wild, with macroscopic characterization and microscopic histopathologies similar to those seen in other vertebrates, including humans. Exposure to carcinogens has confirmed that teleost fish are susceptible to cancer in virtually all organs and tissue types.[59] Several transgenic models have now been developed using tissue-specific expression of human or murine oncogenes, resulting in the development of human-like cancers. These have provided the platform for a second generation of zebrafish screens, which critically include modifier screens for genes or drugs that can affect the onset or progression of oncogene-induced tumors that are genetically based on human molecular oncogenesis. Such modifier screens provide important information for dissecting disease biology and causative pathways, as well as for the identification of new drugs and therapeutic targets. An example of such pathway interactions was demonstrated in zebrafish overexpressing the activated oncogene BRAF in fish melanocytes. These produced pigmented nevi, but when mated to a p53 mutant line, developed fulminant malignant melanoma.[60]

Gene inactivation by homologous recombination as described in yeast, flies, and mice has yet to be successfully performed in the zebrafish, but the challenge of reverse genetics has been met by several other genetic technologies. Transient gene knockdown is possible in zebrafish embryos using morpholinos. Morpholinos are chemically modified antisense oligonucleotides directed either at the translational start site of a gene, blocking protein production, or at a splice site resulting in inappropriate RNA splicing and the formation of nonfunctional proteins. Injection of the morpholino into single-cell–stage embryos results in gene knockdown that is stable for around 4 days, allowing observation of the effects of gene inactivation on embryonic development. This allows rapid assessment of whether gene function in fish creates a phenotype similar to that in mammals.

To provide specific germline gene knockout models, two major technologies have traditionally been employed. Both are based on traditional forward genetic mutagenesis techniques. The first, targeting induced local lesions in genomes (TILLING), combines chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) with an enzyme derived from celery named CELI, which cuts DNA at positions of base pair mismatch. Using this enzyme, pools of genomic DNA from multiple mutagenized fish can be amplified by PCR to identify mutations in a gene of interest. An alternative method is large-scale viral insertional mutagenesis. This technique was pioneered in the laboratory of Dr. Nancy Hopkins and involves the use of a murine retrovirus that inserts randomly into the genome.[61] Both of these technologies are labor intensive and require a large amount of aquarium space. However, novel sequencing technologies that allow rapid identification of mutations are now being used at the Sanger Centre in Cambridge, UK, to sequence ENU mutagenized fish and generate a library of gene-inactivating mutations for every zebrafish gene (http://www.sanger.ac.uk/Projects/D_rerio/zmp). In parallel, the use of targeted genome editing technologies described earlier and shown in Figure 8-3, *B*, is becoming widespread throughout the zebrafish communities.[10] The major advantage of TALEN and zinc-finger nuclease–based strategies for targeted gene knockdown is that they are easily accessible to even small laboratories and, because the location of the double-stranded break is known, this should facilitate strategies to allow homologous recombination to be successfully achieved in this organism.

Although in recent years the fish of choice for modeling tumorigenesis has been the zebrafish, several other teleost

fish have been used for genetic study. The fully sequenced genome of the pufferfish (*Takifugu rubripes*) is particularly of benefit to zebrafish researchers, because there is less evolutionary divergence between fish genes than between those of fish and mammals, and there is less intronic DNA in the pufferfish than in the zebrafish. By comparing the gene localization and sequence in the pufferfish, the few remaining gaps in the now essentially complete zebrafish genome sequence can often be bridged.

One potential drawback when observing tumorigenesis in zebrafish is their stripes. The presence of pigment in the skin of the zebrafish means that after around 5 to 10 days postfertilization (dpf), direct visualization of the developing fish is hampered by the presence of pigment cells. Chemicals such as phenylthiourea are routinely used to delay the onset of pigmentation, but these cannot be used indefinitely, and thus adult fish are not transparent. To facilitate the visualization of adult tissues, a novel pigment mutant has been developed. This mutant fish, aptly named *casper*, is derived from a compound homozygous fish carrying a mutation in the *mitf* gene along with the mutant fish *roy orbison* (whose gene mutation is unknown). This fish remains transparent throughout its life. Thus, fluorescent transgenic markers and in vivo tumor development can be visualized without the necessity to sacrifice the animal (see Figure 8-4, *E*).[62] Furthermore, this model greatly facilitates the study of cell autonomy and transplantation, because transplants into these hosts can be visualized directly in real time.[62,63]

Leukemia Models and Therapy Using Transgenic Zebrafish

The role of the zebrafish as a cancer model combining the attributes of vertebrate biology and model organism genetics came to fruition with the development of T-cell acute lymphoblastic leukemia (T-ALL) in transgenic zebrafish expressing the mouse *c-Myc* oncogene under the lymphoid-specific promoter *rag-2*. The *c-Myc* oncogene was fused to a cDNA encoding the enhanced green fluorescent protein (EGFP), allowing real-time visualization of the leukemic cells. In common with mammalian hematological malignancies, it was possible to sublethally irradiate a recipient wild-type zebrafish and transplant EGFP-positive tumor cells from the *c-Myc*–induced leukemia from another fish by injecting them into the immunosuppressed recipient fish peritoneum. These fish went on to develop leukemia with the same pattern as the donor fish, with initial homing of T lymphoblasts to the thymus gland followed by subsequent infiltration of surrounding tissues and finally dissemination and death.[13] This seminal work demonstrated not only the ability of fish to develop human-like cancers in response to mammalian oncogenes, but also a feasible fish-tumor model system for modifier and drug screens to alter the leukemia phenotype or onset.

Capitalizing on the similarities between human and zebrafish T-cell leukemia as well as the similarities between normal and malignant T-cell development, Ridges and colleagues recently showed the utility of the zebrafish as a model in which to perform in vivo drug screens relevant to human cancers.[64] Based on the fact that treatment of zebrafish embryos with dexamethasone, a drug active in human T-ALL, resulted in a loss of normal thymocytes, the authors used a zebrafish with a green fluorescent thymus to screen a large library of compounds. They identified compounds that led to loss of thymocytes and subsequently prioritized drugs for further assessment by determining whether they had global effects on cell cycle within the fish (which are likely to be generally toxic) and whether the compound had effects in the c-Myc–induced leukemia model. The authors were able to identify one compound (Lenaldekar) that demonstrated specific antitumor effect in human leukemia cells and were able to show that this compound functioned by inhibiting the PI3Kinas/AKT/mTOR pathway, as well as having a tumor-specific effect on cell cycle at the G2/M phase. This study places the zebrafish as a novel in vivo model system in which to identify drugs for cancer treatment.

Zebrafish Screen for Genomic Instability Mutants

Many inherited human syndromes predisposing to cancer (such as Fanconi anemia and Bloom syndrome) are characterized by disruption of genes critical for DNA repair and maintenance of genomic stability. Karyotypic abnormalities are a common finding in the majority of cancers that progressively accumulate over time, highlighting a role for genomic instability in cancer progression. To identify novel genes predisposing to genomic instability and the development of cancer, a forward genetic screen in zebrafish was designed. The screen design used several unique facets of zebrafish genetics. First, wild-type male fish were treated with the mutagen ENU to induce potential *g*enomic *in*stability (gin) mutations per sperm. These fish were mated to fish homozygous for a pigment mutation known as *golden* (gol). Golden embryos in the homozygous state have a characteristic gold-colored pigment in the developing eye, in contrast to wild-type or heterozygous fish, where the pigment is black. In heterozygous golden mutants, an additional recessive mutation predisposing to genomic instability will induce patches of golden pigment as second inactivating mutations occur in the remaining normal golden allele. The number and size of patches of golden tissue can be quantified. This

assay is known as the *mosaic eye* assay.[65] For this assay to effectively identify recessive gin mutations, fish need to be homozygous for the gin mutation (gin/gin) and heterozygous for the gol mutation (gol/+). The progeny from the initial matings are heterozygous for both (gin/+ and gol/+). To obtain this configuration, early-pressure parthenogenesis was used. This technique uses ultraviolet (UV)-irradiated sperm to fertilize the double heterozygous fish, leading to potentially haploid embryos. To maintain a gynogenetic diploid state, the second meiotic division is inhibited by using early pressure applied by a French press. Because of crossing over at the cell cycle stage meiosis I, genes that are nearer to the ends of the chromosomes (telomeric) compared with those nearer to the centromere (centromeric) are more likely to have undergone crossing over; therefore telomeric genes are more likely to be in the heterozygous state. The golden locus is known to be telomeric, and 89% of embryos generated in this way were heterozygous for gol, allowing assessment of genomic instability in the mosaic eye assay. Twelve genomic instability mutants were identified in the screen, all leading to an increased incidence of a variety of cancers in the adult fish in the heterozygous state, but more markedly in the homozygous state. In addition, some of the mutations interacted with one another to produce more severe phenotypes in the double heterozygous state.[66] To date, only preliminary mapping of the mutations has been completed, but identification of the genes causing the observed cancer-predisposing genomic instability phenotypes will likely shed some valuable information on tumor formation in mammals, including humans.

Table 8-1 Internet Resources

http://dbb.urmc.rochester.edu/labs/sherman_f/yeast/Cont.html
http://zfin.org
http://flymove.unimuenster.de/Organogenesis/ImagDiscs/OrgDiscpage.html?http&&&flymove.uni-muenster.de/Organogenesis/ImagDiscs/OrgDiscTxt.html
http://www.neuro.uoregon.edu/k12/zfk12.html
http://www.dnaftb.org/dnaftb
http://zifit.partners.org/ZiFiT
https://boglab.plp.iastate.edu
http://www.sanger.ac.uk/Projects/D_rerio/zmp

Conclusion

This chapter provides the reader an overview of the immense utility and strengths of simple model organisms as tools to dissect the molecular pathogenesis and improve the targeted therapy of human cancer. Model organisms can tell us more about things we know a little of, and reveal to us things of which we know nothing—which is especially important given the emerging complexity of genetic alteration in human cancers. Useful additional Internet resources are provided in Table 8-1.

Key References

1. Bier E. *Drosophila*, the golden bug, emerges as a tool for human genetics. *Nat Rev Genet*. 2005;6:9-23.
3. Buttner S, Ruli D, Vogtle FN, et al. A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. *EMBO J*. 2011;30:2779-2792.
4. Jette CA, Flanagan AM, Ryan J, et al. BIM and other BCL-2 family proteins exhibit cross-species conservation of function between zebrafish and mammals. *Cell Death Differ*. 2008;15:1063-1072.
5. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell*. 2011;21(1):92-101.
8. Voz ML, Coppieters W, Manfroid I, et al. Fast homozygosity mapping and identification of a zebrafish ENU-induced mutation by whole-genome sequencing. *PLoS One*. 2012;7(4):e34671.
9. Bowen ME, Henke K, Siegfried KR, et al. Efficient mapping and cloning of mutations in zebrafish by low-coverage whole-genome sequencing. *Genetics*. 2012 Mar;190(3):1017-1024.
10. Sander JD, Cade L, Khayter C, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol*. 2011 Aug;29(8):697-698.
11. Sander JD, Dahlborg EJ, Goodwin MJ, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods*. 2011 Jan;8(1):67-69.
12. Langenau DM, Feng H, Berghmans S, et al. Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. April 26, 2005;102(17):6068-6073.
13. Langenau DM, Traver D, Ferrando AA, et al. Myc-Induced T cell leukemia in transgenic zebrafish. *Science 2003*. February 7, 2003;299(5608):887-890.
14. Zhu S, Lee JS, Guo F, et al. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. *Cancer Cell*. 2012 Mar 20;21(3):362-373.
16. White RM, Cech J, Ratanasirintrawoot S, et al. DHODH modulates transcriptional elongation in the neural crest and melanoma. *Nature*. 2011 Mar 24;471(7339):518-522.
17. Dar AC, Das TK, Shokat KM, et al. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature*. 2012 Jun 7;486(7401):80-84.
18. Brumby AM, Richardson HE. Using *Drosophila melanogaster* to map human cancer pathways. *Nat Rev Cancer*. 2005 Aug;5(8):626-639.
19. Pierce SE, Fung EL, Jaramillo DF, et al. A unique and universal molecular barcode array. *Nat Methods*. 2006;3(8):601-603.
20. Ooi SL, Pan X, Peyser BD, et al. Global synthetic-lethality analysis and yeast functional profiling. *Trends Genet*. 2006;22(1):56-63.
21. Baetz K, Measday V, Andrews B. Revealing hidden relationships among yeast genes involved in chromosome segregation using systematic synthetic lethal and synthetic dosage lethal screens. *Cell Cycle*. 2006 Mar;5(6):592-595.
22. Tong AHY, Lesage G, Bader GD, et al. Global mapping of the yeast genetic interaction network. *Science*. 2004;303:808-813.
23. Parsons AB, Lopez A, Givoni IE, et al. Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. *Cell*. 2006;126(3):611-625.
25. Krogan NJ, Cagney G, Yu H, et al. Global landscape of protein complexes in the yeast. *Saccharomyces cerevisiae*. *Nature*. 2006;440(7084):637-643.
26. Gavin A-C, Aloy P, Grandi P, et al. Proteome survey reveals modularity of the yeast cell machinery. *Nature*. 2006;440(7084):631-636.
31. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*. 2004;18:2699-2711.
32. Murray AW. Recycling the cell cycle: cyclins revisited. *Cell*. 2004;116(2):221-234.

33. Bayart E, Dutertre S, Jaulin C, et al. The Bloom syndrome helicase is a substrate of the mitotic Cdc2 kinase. *Cell Cycle*. 2006 Aug;5(15):1681-1686.
34. Herr AJ, Ogawa M, Lawrence NA, et al. Mutator suppression and escape from replication error-induced extinction in yeast. *PLoS Genet*. 2011 Oct;7(10): e1002282.
35. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*. 2009 Jul 9;361(2):123-134.
36. Evans CJ, Hartenstein V, Banerjee U. Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. *Dev Cell*. 2003;5(5):673-690.
38. Stewart RA, Li DM, Huang H, et al. A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in *Drosophila*. *Oncogene*. 2003 Sep 25;22(41):6436-6444.
39. Morris EJ, Michaud WA, Ji JY, et al. Functional identification of Api5 as a suppressor of E2F-dependent apoptosis in vivo. *PLoS Genet*. 2006 Nov 17;2(11):e196.
41. Blair SS. Genetic mosaic techniques for studying *Drosophila* development. *Development*. 2003;130:5065-5072.
42. Moreno E, Basler K. dMyc Transforms cells into super-competitors. *Cell*. 2004;117(1):117-129.
43. de la Cova C, Abril M, Bellosta P, et al. *Drosophila* Myc regulates organ size by inducing cell competition. *Cell*. 2004;117(1):107-116.
46. Navarro C, Nola S, Audebert S, et al. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene*. 2005 Jun 23;24(27):4330-4339.
47. Brumby AM, Richardson HE. scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*. *EMBO J*. 2003 Nov 3;22(21):5769-5779.
48. Uhlirova M, Bohmann D. J.N.K.- and Fos-regulated expression Mmp1 cooperates with Ras to induce invasive tumors in *Drosophila*. *EMBO J*. 2006 Nov 15;25(22):5294-5304.
50. Pagliarini RA, Quinones AT, Xu T. Analyzing the function of tumor suppressor genes using a *Drosophila* model. *Methods Mol Biol*. 2003;223:349-382.
51. Massimi P, Gammoh N, Thomas M, et al. HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene*. 2004 Oct 21;23(49):8033-8039.
52. Nakagawa S, Yano T, Nakagawa K, et al. Analysis of the expression and localisation of a LAP protein, human scribble, in the normal and neoplastic epithelium of uterine cervix. *Br J Cancer*. 2004 Jan 12;90(1):194-199.
53. Gardiol D, Zacchi A, Petrera F, et al. Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *Int J Cancer*. 2006;119(6):1285-1290.
60. Patton EE, Widlund HR, Kutok JL, et al. BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr Biol*. 2005;15(3):249-254.
61. Adam Amsterdam TSB. Transgenes as screening tools to probe and manipulate the zebrafish genome. *Dev Dynam*. 2005;234(2):255-268.
62. White RM, Sessa A, Burke C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell*. 2008 Feb 7;2(2):183-189.
63. Feng H, Stachura DL, White RM, et al. T-lymphoblastic lymphoma cells express high levels of BCL2, S1P1, and ICAM1, leading to a blockade of tumor cell intravasation. *Cancer Cell*. 2010 Oct 19;18(4):353-366.
64. Ridges S, Heaton WL, Joshi D, et al. Zebrafish screen identifies novel compound with selective toxicity against leukemia. *Blood*. 2012 Jun 14;119(24):5621-5631.
66. Moore JL, Rush LM, Breneman C, et al. Zebrafish genomic instability mutants and cancer susceptibility. *Genetics 2006*. October 1, 2006;174(2):585-600.

References

1. Bier E. *Drosophila*, the golden bug, emerges as a tool for human genetics. *Nat Rev Genet*. 2005;6:9-23.
2. Lee MG, Nurse P. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature*. 1987;327:31-35.
3. Buttner S, Ruli D, Vogtle FN, et al. A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. *EMBO J*. 2011;30:2779-2792.
4. Jette CA, Flanagan AM, Ryan J, et al. BIM and other BCL-2 family proteins exhibit cross-species conservation of function between zebrafish and mammals. *Cell Death Differ*. 2008;15:1063-1072.
5. Martinou JC, Youle RJ. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell*. 2011;21(1):92-101.
6. Amores A, Force A, Yan Y-L, et al. Zebrafish hox clusters and vertebrate genome evolution. *Science*. 1998;282:1711-1714.
7. Liu TX, Zhou Y, Kanki JP, et al. Evolutionary conservation of zebrafish linkage group 14 with frequently deleted regions of human chromosome 5 in myeloid malignancies. i2002 Apr 30; 99(9): 6136–6141.
8. Voz ML, Coppieters W, Manfroid I, et al. Fast homozygosity mapping and identification of a zebrafish ENU-induced mutation by whole-genome sequencing. *PLoS One*. 2012;7(4):e34671.
9. Bowen ME, Henke K, Siegfried KR, et al. Efficient mapping and cloning of mutations in zebrafish by low-coverage whole-genome sequencing. *Genetics*. 2012 Mar;190(3):1017-1024.
10. Sander JD, Cade L, Khayter C, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol*. 2011 Aug;29(8):697-698.
11. Sander JD, Dahlborg EJ, Goodwin MJ, et al. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat Methods*. 2011 Jan;8(1):67-69.
12. Langenau DM, Feng H, Berghmans S, et al. Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. April 26, 2005;102(17):6068-6073 2005.
13. Langenau DM, Traver D, Ferrando AA, et al. Myc-Induced T cell leukemia in transgenic zebrafish. *Science 2003*. February 7, 2003;299(5608):887-890.
14. Zhu S, Lee JS, Guo F, et al. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. *Cancer Cell*. 2012 Mar 20;21(3):362-373.
15. Bjornsti MA. Cancer therapeutics in yeast. *Cancer Cell*. 2002 Oct;2(4):267-273.
16. White RM, Cech J, Ratanasirintrawoot S, et al. DHODH modulates transcriptional elongation in the neural crest and melanoma. *Nature*. 2011 Mar 24;471(7339):518-522.
17. Dar AC, Das TK, Shokat KM, et al. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature*. 2012 Jun 7;486(7401):80-84.
18. Brumby AM, Richardson HE. Using *Drosophila melanogaster* to map human cancer pathways. *Nat Rev Cancer*. 2005 Aug;5(8):626-639.
19. Pierce SE, Fung EL, Jaramillo DF, et al. A unique and universal molecular barcode array. *Nat Methods*. 2006;3(8):601-603.
20. Ooi SL, Pan X, Peyser BD, et al. Global synthetic-lethality analysis and yeast functional profiling. *Trends Genet*. 2006;22(1):56-63.
21. Baetz K, Measday V, Andrews B. Revealing hidden relationships among yeast genes involved in chromosome segregation using systematic synthetic lethal and synthetic dosage lethal screens. *Cell Cycle*. 2006 Mar;5(6):592-595.
22. Tong AHY, Lesage G, Bader GD, et al. Global mapping of the yeast genetic interaction network. *Science*. 2004;303:808-813.
23. Parsons AB, Lopez A, Givoni IE, et al. Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast. *Cell*. 2006;126(3):611-625.
24. Pandey A, Mann M. Proteomics to study genes and genomes. *Nature*. 2000;405(6788):837-846.
25. Krogan NJ, Cagney G, Yu H, et al. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature*. 2006;440(7084):637-643.
26. Gavin A-C, Aloy P, Grandi P, et al. Proteome survey reveals modularity of the yeast cell machinery. *Nature*. 2006;440(7084):631-636.
27. Paul N. The Josef Steiner Lecture: CDKs and cell-cycle control in fission yeast: relevance to other eukaryotes and cancer. *Int J Cancer*. 1997;71:707-708.
28. Hartwell LH. Synchronization of haploid yeast cell cycles, a prelude to conjugation. *Exp Cell Res*. 1973;76(1):111-117.
29. Duntze W, MacKay V, Manney TR. *Saccharomyces cerevisiae*: a diffusible sex factor. *Science*. 1970;168:1472-1473.
30. Hartwell LH. Nobel Lecture. Yeast and cancer. *Biosci Rep*. 2002;22:373-394.
31. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*. 2004;18:2699-2711.
32. Murray AW. Recycling the cell cycle: cyclins revisited. *Cell*. 2004;116(2): 221-234.
33. Bayart E, Dutertre S, Jaulin C, et al. The Bloom syndrome helicase is a substrate of the mitotic Cdc2 kinase. *Cell Cycle*. 2006 Aug;5(15):1681-1686.
34. Herr AJ, Ogawa M, Lawrence NA, et al. Mutator suppression and escape from replication error-induced extinction in yeast. *PLoS Genet*. 2011 Oct;7(10): e1002282.
35. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*. 2009 Jul 9;361(2):123-134.
36. Evans CJ, Hartenstein V, Banerjee U. Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. *Dev Cell*. 2003;5(5):673-690.
37. Potter CJ, Turenchalk GS, Xu T. *Drosophila* in cancer research: an expanding role. *Trends Genet*. 2000;16(1):33-39.
38. Stewart RA, Li DM, Huang H, et al. A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in *Drosophila*. *Oncogene*. 2003 Sep 25;22(41):6436-6444.
39. Morris EJ, Michaud WA, Ji JY, et al. Functional identification of Api5 as a suppressor of E2F-dependent apoptosis in vivo. *PLoS Genet*. 2006 Nov 17;2(11):e196.
40. Staehling-Hampton K, Ciampa PJ, Brook A, et al. A genetic screen for modifiers of E2F in *Drosophila melanogaster*. *Genetics*. 1999;153:275-287.
41. Blair SS. Genetic mosaic techniques for studying *Drosophila* development. *Development*. 2003;130:5065-5072.
42. Moreno E, Basler K. dMyc Transforms cells into super-competitors. *Cell*. 2004;117(1):117-129.
43. de la Cova C, Abril M, Bellosta P, et al. *Drosophila* Myc regulates organ size by inducing cell competition. *Cell*. 2004;117(1):107-116.
44. Johnston LA, Prober DA, Edgar BA, et al. *Drosophila* myc regulates cellular growth during development. *Cell*. 1999;98(6):779-790.
45. Bilder D, Li M, Perrimon N. Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors. *Science*. 2000 Jul 7;289(5476):113-116.
46. Navarro C, Nola S, Audebert S, et al. Junctional recruitment of mammalian Scribble relies on E-cadherin engagement. *Oncogene*. 2005 Jun 23;24(27):4330-4339.
47. Brumby AM, Richardson HE. scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*. *EMBO J*. 2003 Nov 3;22(21):5769-5779.
48. Uhlirova M, Bohmann D. J.N.K.- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in *Drosophila*. *EMBO J*. 2006 Nov 15;25(22):5294-5304.
49. St Johnston D. The art and design of genetic screens: *Drosophila melanogaster*. *Nat Rev Genet*. 2002 Mar;3(3):176-188.
50. Pagliarini RA, Quinones AT, Xu T. Analyzing the function of tumor suppressor genes using a *Drosophila* model. *Methods Mol Biol*. 2003;223:349-382.
51. Massimi P, Gammoh N, Thomas M, et al. HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene*. 2004 Oct 21;23(49):8033-8039.
52. Nakagawa S, Yano T, Nakagawa K, et al. Analysis of the expression and localisation of a LAP protein, human scribble, in the normal and neoplastic epithelium of uterine cervix. *Br J Cancer*. 2004 Jan 12;90(1):194-199.
53. Gardiol D, Zacchi A, Petrera F, et al. Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *Int J Cancer*. 2006;119(6):1285-1290.
54. Moberg KH, Bell DW, Wahrer DCR, et al. Archipelago regulates cyclin E levels in *Drosophila* and is mutated in human cancer cell lines. *Nature*. 2001;413(6853):311-316.
55. Koepp DM, Schaefer LK, Ye X, et al. Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science 2001*. October 5, 2001;294(5540):173-177.
56. Nusslein-Volhard C. General Motors Cancer Research Prizewinner Laureates Lectures. Alfred P. Sloan, Jr. Prize. The formation of the embryonic axes in *Drosophila*. *Cancer*. 1993 May 15;71(10):3189-3193.

57. Haffter P, Granato M, Brand M, et al. The identification of genes with unique and essential functions in the development of the zebrafish *Danio rerio*. 1996:1-36.
58. Mullins MC, Hammerschmidt M, Haffter P, et al. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol*. 1994;4(3):189-202.
59. Amatruda JF, Shepard JL, Stern HM, et al. Zebrafish as a cancer model system. *Cancer Cell*. 2002;1(3):229-231.
60. Patton EE, Widlund HR, Kutok JL, et al. BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma. *Curr Biol*. 2005;15(3):249-254.
61. Adam Amsterdam TSB. Transgenes as screening tools to probe and manipulate the zebrafish genome. *Dev Dynam*. 2005;234(2):255-268.
62. White RM, Sessa A, Burke C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell*. 2008 Feb 7;2(2):183-189.
63. Feng H, Stachura DL, White RM, et al. T-lymphoblastic lymphoma cells express high levels of BCL2, S1P1, and ICAM1, leading to a blockade of tumor cell intravasation. *Cancer Cell*. 2010 Oct 19;18(4):353-366.
64. Ridges S, Heaton WL, Joshi D, et al. Zebrafish screen identifies novel compound with selective toxicity against leukemia. *Blood*. 2012 Jun 14;119(24):5621-5631.
65. Streisinger G. Attainment of minimal biological variability and measurements of genotoxicity: production of homozygous diploid zebra fish. *Natl Cancer Inst Monogr*. 1984 May;65:53-58.
66. Moore JL, Rush LM, Breneman C, et al. Zebrafish genomic instability mutants and cancer susceptibility. *Genetics 2006*. October 1, 2006;174(2):585-600.

Monte M. Winslow and Tyler Jacks

9

Genetic Mouse Models of Cancer

The study of many different organisms has contributed to our understanding of cancer at the molecular, cellular, and organismal levels. Considerable effort is focused on the rational design and use of mouse models, including spatially and temporally controlled genetic modifications to recapitulate human cancers. Long before the development of genetically engineered animal models, research on mice, rats, rabbits, and chickens led to major discoveries directly related to cancer, including the discovery of oncogenes and the biochemical purification of tumor suppressor proteins.[1-4] In addition, many key regulators of proliferation, differentiation, and cell death have been characterized by studying developmental processes in mice. The knowledge of pathways that regulate organ development is an important framework on which to build our understanding of all aspects of tumor initiation, progression, and metastasis.

In this chapter, we discuss mouse models of cancer, emphasizing the techniques used to create genetically engineered mouse models and the application of these models to cancer research. Several fundamental discoveries resulting from the use of mouse models are also highlighted, as well as the important role of these models in the future of cancer research.

Basis for Mouse Models of Cancer

Our understanding of the genetic alterations in human tumors and the ability to manipulate the mouse genome has allowed for the development of models of human cancer.[5-8] Mice are the preferred model organism with which to study the complex processes of tumor development and progression for many reasons, including their short generation time, small size, availability of inbred strains, and the close genetic relationship between mice and humans. Fish, flies, and worms have also been successfully used to investigate tumorigenesis, and the unique genetic tools available in these species have allowed for a range of informative experiments to be performed.[9-12]

Observational and correlative studies of human cancer combined with in vitro experiments on human cancer cell lines have contributed a great deal to the foundation of our knowledge of tumorigenesis. The dissection of cancer development and progression in humans is greatly limited by the difficulty in accessing lesions at various stages of development and the inability to test gene function in vivo except by pharmacologic means. The interrogation of gene function in vitro is limited to genes that control the intrinsic processes of cancer cells, including proliferation, differentiation, and cell death. Moreover, the complex interactions between different cell types within the tumor are poorly recapitulated in vitro, and the selective pressure of in vitro growth may significantly alter the genotype and phenotype of cultured cells. For all these reasons, animal models in which the entire developmental progression of the disease from tumor initiation to metastatic outgrowth occur in vivo are of paramount importance.

The underlying genetic heterogeneity of the human population, the existence of subtypes of different malignancies, and the genetic and genomic heterogeneity within tumors of the same type complicate studies of human tumor gene expression and mutational analysis. The induction of tumors with specific oncogenic alterations in mice on inbred backgrounds can overcome many of these limitations. Mouse models also offer the ability to assign causality to genetic alteration and to assess the roles of certain genes and pathways in vivo.

Mouse Models of Cancer

Modeling human cancer in mice has evolved as techniques to modify the mouse genome have been developed. Combinations of the approaches described in the following sections have been used to model many human cancers. The plethora of options to create these models has been used to address many fundamental questions in tumor biology.

Spontaneous and Mutagen-Induced Tumor Models

Mice spontaneously develop a spectrum of cancers. The observation that different inbred strains of mice develop cancer at different frequencies gave early support to the idea that the genetic background of a mouse (or person) can predispose them to cancer. Spontaneous tumor formation is often assessed in non-genetically engineered mouse lines to determine whether the specific gene mutation influences the prevalence, progression, or types of cancers that arise (Figure 9-1, *A*).

Many known or suspected carcinogens have been used to create mouse models of cancer (see Figure 9-1, *B*). These models rely on the treatment of mice with chemical or physical mutagens, which most often leads to the development of genetically undefined cancers. Carcinogen-induced cancer protocols can be used with genetic techniques to create combined carcinogen/genetic models of human cancer.

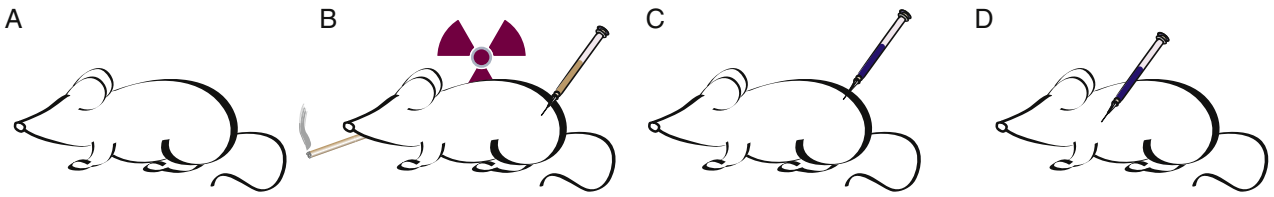
Xenograft and Orthotopic Models

The transplantation of human and mouse tumor cells into recipient mice has been used extensively to investigate tumor development in vivo (see Figure 9-1, *C* and *D*). Human tumor cell lines can be injected orthotopically into the organ from which the tumor originated, intravenously (to mimic the metastatic spread of cancer cells) or subcutaneously (to simply allow the tumor to grow in vivo). Mouse tumor cell lines can be transplanted into syngeneic immunocompetent recipients, whereas human cell lines must be transplanted into immunocompromised recipients. This in vivo tumor growth requires many of the proper tumor-host interactions, including the development of vasculature and recruitment of supportive stromal cells. However, these procedures often involve the injection of high numbers of cells and do not recapitulate the series of events that lead to human cancer. Nonetheless, the ability to manipulate tumor cell lines in vitro before transplantation and the speed and reproducibility of tumor growth are major advantages of these approaches.

Genetically Engineered Mouse Models

Gene expression and genomic analyses of human cancers have uncovered many of the important genetic changes in different tumor types. The knowledge gleaned from these studies coupled with the ability to create germline and somatic alterations in the mouse genome has allowed the creation of genetically defined mouse models of cancer that approximate human cancer at the genetic and histologic levels. Transgenic overexpression was the first genetic technology used to create mouse tumor models[13-15] (Figure 9-2, *A*). Tissue-specific promoters can be used to drive the expression of genes of interest in the desired cell type or tissue, and the tumorigenic consequences can be determined. More elaborate transgenic approaches also allow transgene expression to be controlled temporally.

An interesting system for the delivery of genes to somatic cells in vivo uses avian retroviral vectors. Transgenic



	SPONTANEOUS	CARCINOGEN-INDUCED	XENOGRAFT TRANSPLANT	ORTHOTOPIC TRANSPLANT
	Wait for untreated mice to get tumors with age.	Expose mice to known or suspected carcinogens.	Inject human tumor cells into mice. Often cells are injected subcutaneously.	Inject tumor cells into their tissue of origin.
Pros	Tumors arise in proper tissue environment.	Use carcinogens known to promote tumor formation in humans.	Use human tumor cells. Tumor cells can be manipulated in vitro prior to injection. Rapid generation time.	Use human or mouse tumor cells. Tumor cells can be manipulated in vitro prior to injection. Tumors arise in proper tissue environment.
Cons	Long latency, unspecified tumor development. Unknown genetic alteration in tumors.	Unknown genetic alteration in tumors. Unsynchronized and variable tumor development.	Incompatibility between human and mouse growth factors. Requires the use of immunocompromised recipients. Often use tumor cell lines. Injection of high numbers of cells. Growth in inappropriate tissue.	Not all tissues are amenable to orthotopic injections. Often use tumor cell lines. Injection of high numbers of cells.

Figure 9-1 Non–genetically engineered mouse models of cancer **(A,B)** Mice develop tumors spontaneously or in response to carcinogen exposure. **(C,D)** Transplantation of human or mouse tumor cells into recipient mice provides a rapid method to study cell growth and progression in vivo.

expression of the cell surface receptor for the RCAS virus (tva) allows the specific and stable infection of a cell type of interest[16] (see Figure 9-2, *B*). The viral vectors can be engineered to express a gene of interest, and the effect of these genes on tumorigenesis can be determined after in vivo infection of the tva-expressing permissive cell type.

Techniques to alter the germline of mice allow the deletion or alteration of genomic loci (see Figure 9-2, *C* and *D*). These alterations can also be induced in cell-type and temporally regulated fashions. Such powerful approaches allow mouse models to be created that mimic the loss of tumor suppressor genes and activation of oncogenes that occur in different human cancers, resulting in mouse models that closely resemble the human disease. These genetically engineered mouse models are being used in a myriad of research settings to further our understanding of tumor biology.

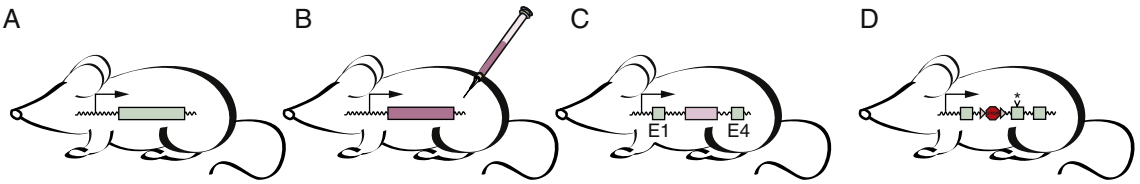
Techniques to Modify the Mouse Genome

Different genetic strategies can be used to overexpress, alter, or reduce the expression of genes that affect tumor incidence or progression. Genetic mouse models begin to recapitulate the selected human cancer when the genetic alterations are consistent with those detected in human cancers and when those alterations produce a tumor lesion that appears histologically similar to the human disease. Transgenic overexpression, induced and germline gene deletion, and conditional expression of activated oncogenes allow most of the genetic alterations found in human cancers to be modeled in mice.

Transgenic Mice

Transgenic mice have an extra copy of the gene of interest controlled by a ubiquitous or tissue-specific promoter (Figure 9-3, *A*). The use of a cell-type–specific promoter provides spatial control over the expression of the transgene. A normal or mutant form of a gene can be overexpressed to ascertain its effect on tumor development. In addition to gene overexpression, transgenic mice can also be used to reduce gene expression or protein function. The expression of dominant negative or viral proteins that interfere with endogenous protein function has been used to assess the effect of disrupting certain pathways on in vivo tumorigenesis. In addition, RNA interference (RNAi) can be used to reduce the expression of a gene of interest in mice (see Figure 9-3, *B*).[17,18]

Traditional transgenic mice constitutively express the transgene in the chosen cell type, potentially disrupting organ development or tissue homeostasis. Therefore, systems have



	Transgenic gene overexpression	RCAS viral-based gene expression system	Germline genetic alteration	Spatial and temporal control of oncogenes and tumor suppressors
	Tissue-specific expression of a gene of interest	Specific in vivo gene transfer to cells expressing the viral TVA receptor by an avian retrovirus	Deletion or alteration of gene of interest in the germline.	Alteration of gene of interest in a cell-type-specific and temporally controlled manner to assess its role in tumorigenesis
Pros	Recapitulates overexpression or amplification of genes in human tumors. Tissue-specific expression.	Transfers genes into somatic cells. Can control timing of tumor initiation. Confines expression to cell type of interest. Can express multiple genes.	Model familial cancer syndromes. Tumors arise in proper tissue environment.	Can control timing of tumor initiation. Confine expression to cell type of interest. Intact immune system. Tumors arise in proper tissue environment. Known genetic alterations.
Cons	Transgene expression is affected by integration site. Inability to recreate gene mutation or true gene loss.	Requires production of TVA transgenic mouse lines. Virus can only infect dividing cells. Potential effect of viral insertion into the genome.	Unknown additional genetic alteration required for tumor formation.	Requires production of genetically altered mouse lines. Requires prior knowledge of oncogenic lesion in specific tumor type.

Figure 9-2 Genetically modified mouse models of cancer **(A,B)** Transgenic gene expression and **(C,D)** the alteration of endogenous loci allow induction of tumors in mice with genetic alterations analogous to those in human cancer.

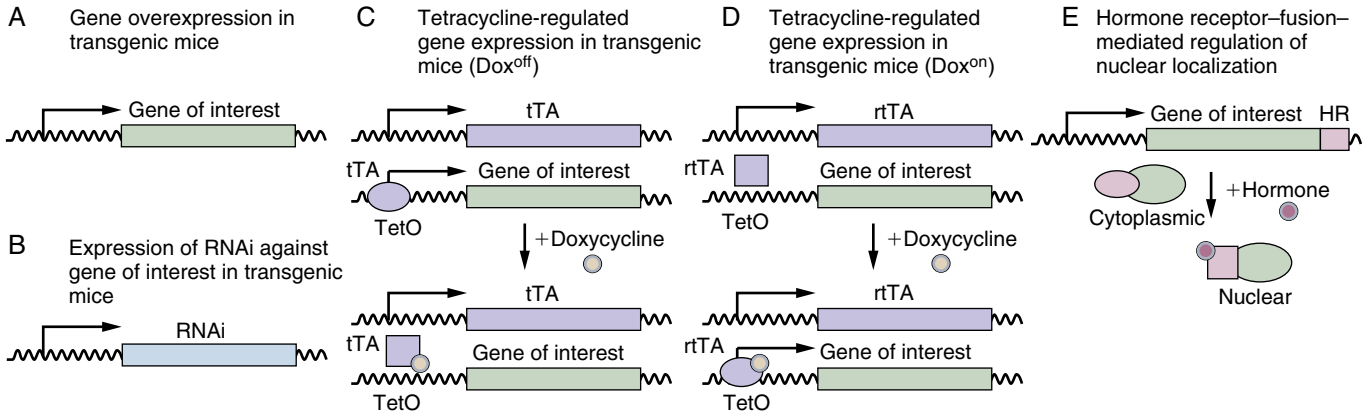


Figure 9-3 The toolbox for the transgenic control of gene expression and protein function in genetically modified mice (A,B) The use of tissue-specific promoters allows the expression of a gene or interfering RNA of interest in the desired tissue. **(C,D)** Regulation of gene expression by the tetracycline system adds a level of temporal control based on a change in conformation of the tetracycline transactivator (tTA) or reverse tetracycline transactivator (rtTA) in the presence of doxycycline. **(E)** The expression of hormone receptor (HR)-fusion proteins allows the nuclear translocation of proteins of interest only in the presence of the hormone.

been developed to allow the temporal control of transgene expression or function. Two complementary systems rely on a tetracycline-dependent transactivation to control the spatial and temporal expression of the gene of interest[19-21] (see Figure 9-3, *C* and *D*). The tetracycline transactivator (tTA) drives the expression of genes under the control of the bacterial tetracycline-dependent operator (tetO). The transactivation function of the tTA is blocked when tetracycline derivatives, often doxycycline, are present (see Figure 9-3, *C*). The reverse tTA (rtTA) works analogously to tTA, except that the expression of the tetO-controlled gene is induced only in the presence of doxycycline (see Figure 9-3, *D*). Exposure of mice with cell-type–specific expression of the tTA or rtTA transgene and a tetO-controlled gene of interest to doxycycline can be used to turn gene expression on and off. These systems have allowed investigators to control tumor initiation and evaluate the requirement for continued oncogene expression during tumor maintenance and progression.[22-28]

The fusion of oncogenes and tumor suppressors to hormone receptors has also been used to regulate protein function by controlling their subcellular localization (see Figure 9-3, *E*). In-frame fusion of a gene of interest to the estrogen receptor (ER) or a truncated progesterone receptor (APR) creates a fusion protein that is sequestered in the cytoplasm until the cell is exposed to the appropriate hormone that induces its nuclear import (see Figure 9-3, *E*). Modified ERs (ER(TAM) and ER(T2)) have been created that translocate to the nucleus in the presence of 4-hydroxytamoxifen but not natural ER ligands, thus reducing background translocation.[29,30] These acutely switchable protein alleles have been used to determine the execution point for various nuclear proteins, including oncogenes and tumor suppressors.[31-33]

Gene-Targeted Mice

The ability to alter endogenous loci within the mouse genome has dramatically affected every field of biology.[34] Homologous recombination in embryonic stem cells allows the specific deletion or alteration of genomic loci (Figure 9-4, *A* and *B*). This technique was initially used by cancer biologists to make germline deletions of several genes implicated in human cancer.[35-38] These conventional "knockout mice" lack the gene of interest in every cell in the animal. Germline deletion of some genes results in embryonic lethality, necessitating the analysis of heterozygous mutant mice or the use of conditional deletion strategies. Several tumor suppressor genes are mutated in the germlines of families, predisposing them to cancer, and mice with heterozygous deletion or mutation of these genes can serve as useful models to study tumor development under these sensitizing genetic conditions.[39-43]

The ability to delete genes specifically in a chosen cell type is comparable to the use of tissue-specific promoters to drive transgene expression (see Figure 9-4, *C*). Using bacteriophage-derived Cre recombinase, it is possible to delete genomic regions flanked by loxP nucleotide sequences (these loci are referred to as *floxed*).[34,44] FLPe recombinase is used less frequently but can also be used to recombine loci flanked by FRT sequences (see Figure 9-4, *C*).

The development of mice that express Cre recombinase in defined cell types and the creation of floxed alleles of many important cancer genes have allowed researchers to investigate the role of these genes in the development of various types of tumors in a highly controlled manner. Cre recombinase can also be used to induce chromosomal translocations analogous to the translocations that are pathognomonic of certain hematopoietic cancers[45,46] (see Figure 9-4, *D*).

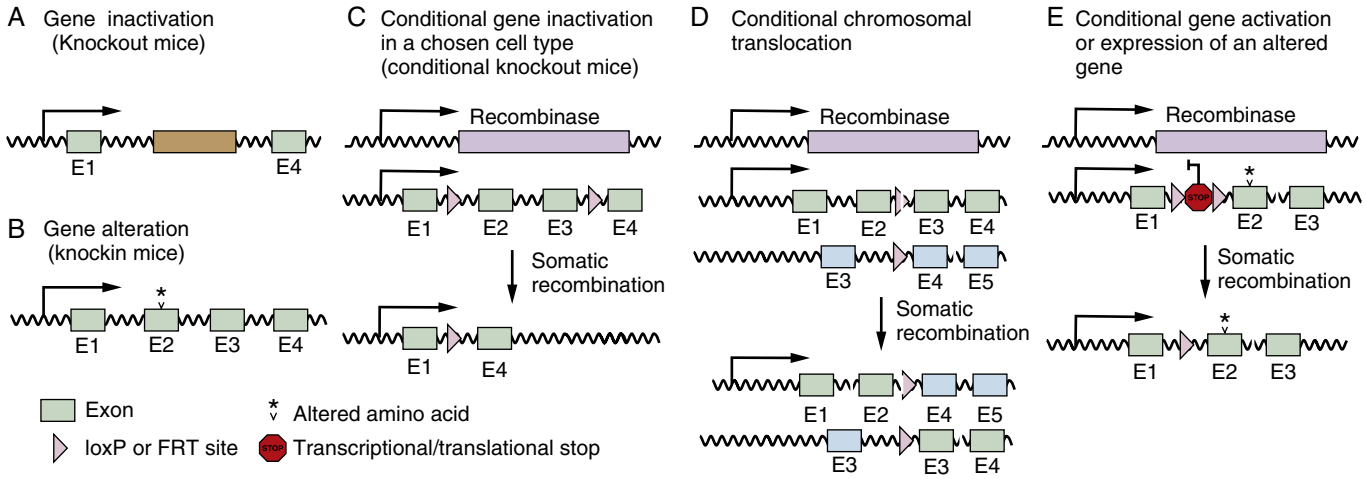


Figure 9-4 The toolbox for the deletion or genetic modification of endogenous genes in mice Genetic alteration of endogenous loci to inactivate **(A),** alter **(B)** or conditionally activate **(D,E),** or inactivate **(C)** genes. Homologous recombination allows the deletion or alteration of gene coding sequences. **(C-E)** The expression of a recombinase (Cre or FLPe) from a tissue-specific promoter or virus allows the spatially restricted deletion **(C),** translocation **(D),** or induced expression **(E)** of a targeted allele.

The expression of activated oncogenes is an important aspect of mouse models of human cancer. To express a mutated oncogene at its physiologic level from its endogenous promoter (as is the case in most human cancers), mice have been engineered with a floxed transcription/translation stop cassette in the first exon of a chosen mutant oncogene. These oncogenes remain silent until Cre-recombinase removes the stop cassette, allowing the expression of the mutant oncogene in the chosen cell type (see Figure 9-4, *E*).

Specific promoters direct the expression or deletion of genes to a desired cell lineage, and sophisticated systems can also allow the timing of gene alteration to be controlled. In these situations, however, every cell of the chosen cell type undergoes the same oncogenic event, which is in stark contrast to the initiation of human tumors where a single cell likely incurs the oncogenic alteration. Although inducing these genetic changes in a single cell may not be the most appropriate approach in experimental research, the use of viruses to deliver Cre recombinase to a subset of cells may be an acceptable medium. In these systems, viruses (often adenoviral or lentiviral vectors) are used to deliver Cre to a fraction of the cells in the organ of interest in mice that are genetically poised to express or delete genes of interest. These viral vectors have been used to initiate multifocal non–small-cell and small-cell lung cancer, hepatocellular carcinoma, ovarian cancer, and various brain tumors.[47-51]

Rational creativity may be the underlying theme of these mouse models. Table 9-1 contains a selection of mouse models that use a variety of different genetic techniques to model different tumor types. As our knowledge of the genetic alterations in human cancers increases, our ability to control their expression in mice will also expand with the application of additional orthogonal systems.

Applications of Mouse Models to Cancer Biology

Combinations of the methods described in the preceding sections have been used to address several important questions in cancer biology, including oncogene addiction and the cooperation and interdependence of various oncogenes and tumor suppressors.

Cross-Species Comparisons

The comparison of tumors from different species has highlighted the central role for several oncogenic and tumor suppressor pathways. Mutations in *p53* are found in about half of human tumors, but *p53* is also mutated in tumors in the soft-shell clam, *Mya arenaria*, underscoring the importance of this tumor suppressor and the conservation of critical alterations across diverse phyla.[52,53] Cross-species comparison of gene expression and genomic changes in tumors from mice and humans has also yielded valuable insight into the important genetic changes in cancer.[54-58] The genetic changes in human tumors are often complex and are overlaid on the considerable allelic variation among individuals. Although possible, pinpointing the important somatic changes or genomic alterations can become unwieldily complex.[59] By comparing the overlapping genomic and genetic changes in mouse and human tumors of the same type (and even tumors containing several of the same oncogenic events), the minimal critical genetic changes can be established. In addition, these changes can be functionally validated in the mouse models that aided in their identification.

Table 9-1 Examples of Genetically Modified Mouse Models of Cancer

Tumor Type	Genetic Modification	Mouse Model	Common Alterations in Human Cancer	Reference
Breast cancer (Chapter 36)	Transgenic expression of an oncogene	MMTV-*HER2*	*HER2*, *C-MYC*, and/or *cyclin D1* amplification; germline *BRCAL* or *BRCA2* mutations; *p53*, *RB1*, and/or *PTEN* loss	93,94
Prostate cancer (Chapter 38)	Transgenic expression of the SV40 large T-antigen to block tumor suppressors	Pb-*T antigen*	*RB*, *p53*, *PTEN*, and/or *NKX3.1* loss; active K-RAS, active H-RAS	95
Acute lymphoblastic leukemia (Chapter 26)	Tetracycline-regulated oncogene expression (Dox off)	*EμSRα-tTA; tetO-cMYC*	Immunoglobulin *locus-MYC* translocation	24
Melanoma (Chapter 42)	Tetracycline-regulated oncogene expression (Dox on) in the absence of a tumor suppressor locus	*Tyr-rtTA; tetO-Hras^{G,2V}; Ink4a/Arf$^{-/-}$*	*INK4a/ARF* loss; N-RAS activation, B-RAF activation; *PTEN* loss; *MITF* amplification, *NEDD9* amplification	26
Pancreatic B-cell adenocarcinoma (Chapter 35)	Estrogen receptor–oncogene fusion regulated by tamoxifen and transgenic expression of a prosurvival gene	pIns-*cMycER^{TAM}*; RIP$_7$-*Bclx$_L$* with tamoxifen	*MEN$_1$* loss	32
Glioblastoma (Chapter 40)	Avian virus delivered oncogene in the absence of a tumor suppressor locus	Nestin-*tva; Ink4a/Arf$^{-/-}$* with RCAS-EGFR*	*INK4a/ARF* loss; *EGFR* amplification; *p53* loss, *RB$_1$* loss; *CDK4* amplification	16
Colon cancer (Chapter 34)	Mutation of a tumor suppressor gene	*APC$^{min/+}$; APC$^{Δ716/+}$; APC$^{1638N/+}$*	*APC*, *SMAD4*, and/or *p53* loss; active K-RAS, active N-RAS	96-98
Small-cell lung cancer (Chapter 32)	Deletion of two tumor suppressor genes with viral-Cre	*P53$^{flox/ftox}$; Rb$^{flox/ftox}$* with viral-Cre	*RB$_1$* loss and *p53* loss; *N-Myc* or *L-MYC* amplification	50
Acute myeloid leukemia (Chapter 28)	Conditional chromosomal translocation	*Mll$^{loxp/+}$; Enl^{loxp}/+; Lmo2$^{Cre/+}$*	Many different translocations, including the *MLL-ENL* translocation	44
Pancreatic ductal adenocarcinoma (Chapter 35)	Conditional activation of an endogenous oncogene and expression of a point mutant tumor suppressor gene	*Kras$^{LsL-G12D/+}$; p53$^{LsL-R172H/+}$;* Pdx1-Cre	Active K-RAS; *p53* loss, *SMAD4* loss	99
Non–small-cell lung cancer (Chapter 32)	Conditional activation of an endogenous oncogene and deletion of a tumor suppressor gene with viral-Cre	*Kras$^{LSL-G12D/+}$; p53$^{flox/flox}$* with viral-Cre	Active K-RAS; *p53* loss; *EGFR* activation and amplification	100
Head and neck squamous-cell carcinoma (Chapter 33)	Progesterone-regulated conditional activation of an endogenous oncogene and deletion of a tumor suppressor gene	*Kras$^{LSL-G12D/+}$; TGFβRII$^{flox/flox}$; K5-CrePR$_1$* with RU486	*p53* loss, *Ink4a/Arf* loss, *cyclin D$_1$* amplification, active K-RAS, active H-RAS	101

Oncogene Addiction

Mutation or overexpression of oncogenes can initiate tumor development. The use of tetracycline-regulated expression systems has documented the requirement for continued oncogene expression for growth and survival of established tumors and metastases.[22-28] Although most tumors undergo dramatic cell death and regress after oncogene inactivation, the regression is not always complete, and tumor subclones that escape the requirement for the initiating oncogene can recur (Figure 9-5).[55] Interestingly, in a model of MYC-induced hepatocellular carcinoma, MYC reactivation after tumor regression results in the development of tumors that are clonally related to the initial primary tumor, indicating that dormancy can also be a result of oncogene inactivation.[25] These dramatic results validate the future use of these models to predict the outcome of altering specific pathways predicted to influence tumor survival or progression. Clinically, pharmacologic oncogene inactivation can successfully reduce tumor growth, supporting the concept of oncogene addiction. In particular, a subset of non–small-cell lung cancer with mutant EGFR,[60,61] gastrointestinal stromal tumors with active/mutant c-Kit,[62] and chronic myeloid leukemia with the BCR-ABL translocation[63] have been successfully treated with small molecules targeting these driving oncogenes.

Oncogene Cooperation and Codependence

The hypothesis of a multistep model of tumorigenesis mediated by multiple genetic alterations raises the interesting question of how these genes cooperate to promote tumor development. In vitro studies in immortalized cell lines and primary fibroblasts were initially used to show the cooperativity of different oncogenes.[64] The tumor suppressor networks, the relationship between oncogenes and their target genes, and the cooperation of different genetic changes in promoting tumor initiation and progression have also been studied in vivo using genetic methods.[65-67]

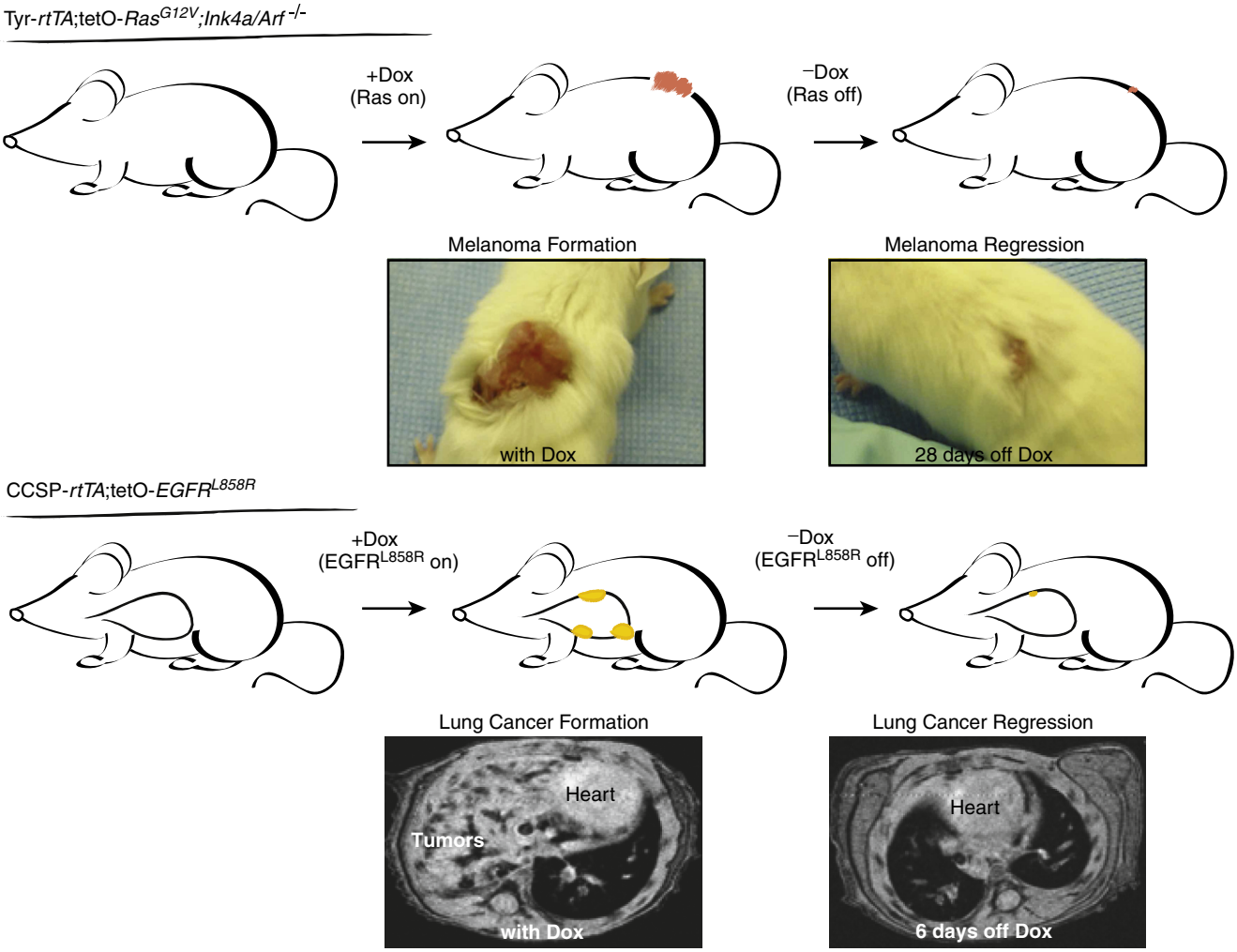


Figure 9-5 Oncogene addiction of mouse tumors predicts the outcome of therapeutic blockade of oncogene expression or activity
Melanocyte-specific oncogenic Ras expression leads to the formation of melanoma, which regresses when Ras is no longer expressed. Lung epithelial expression of an active point mutant of EGFR produces lung adenocarcinoma development. The maintenance of these lung tumors relies on the continued expression of the oncogene. *Melanoma images are courtesy Joseph Gans and Lynda Chin, Dana-Farber Cancer Institute, Harvard University. Lung adenocarcinoma images are courtesy Katerina Politi and Harold Varmus, Memorial Sloan-Kettering Cancer Center.)*

Genetic epistasis experiments in mice have identified several critical targets of specific oncogenes that mediate different aspects of tumorigenesis.[33,65,68] Moreover, genes that enhance or reduce the effect of tumor suppressor gene mutation and oncogene expression have also been identified.[66,67,69]

Future Directions of Cancer Models

The most advanced genetically engineered mouse models of human cancer reflect their human counterparts at the genetic and histologic levels. These mouse models are now poised to lead the way to the discovery of new genes and pathways dysregulated in cancer and aid in the development and screening of potential therapeutics.

In Vivo Screens

Transposon and retroviral insertional mutagenesis, short-interfering RNA (siRNA) libraries, and advances in the analysis of gene expression and genomic alteration allow mouse models to be used as tools in the discovery of new cancer genes and pathways. Each of these approaches has been used to identify genes that promote tumorigenesis.[70-79] Unlike chemical or physical mutagens, insertional mutagens allow the identification of mutated genes. By using these mutagens in sensitized backgrounds (e.g., loss of a tumor suppressor or expression of an oncogene), the genes that regulate tumor initiation, invasion, or metastasis can be identified. Whole-genome or focused siRNA and shRNA library screens for genes that influence transformation have been conducted in vitro[74-76] and in vivo.[80-82] Genes discovered by these methods can be confirmed in the

same tumor model in which they are found, and these unbiased approaches have begun to contribute to the identification of genes and pathways that are potential therapeutic targets.

Validation of Pharmaceutical Targets and Preclinical Trials

The development of new therapeutics requires carefully designed preclinical studies in models that most closely approximate human disease. Xenograft tumor models are the mainstay of current preclinical testing. Although several obstacles must be overcome before genetic mouse models can fully reach their potential in pharmacologic and biotechnological settings, these models may more accurately reflect the therapeutic response of patients.[7,8,83,84] The use of genetically defined mouse models may prioritize potential therapeutic compounds and stratify patients based on the mutational status of their tumors.[85] These efforts should accelerate the translation of novel therapies into the clinic.

Biomarkers for Early Tumor Detection

The detection of cancer at an early stage is of paramount importance, as patients diagnosed with early-stage disease invariably have a better prognosis. Unfortunately, there is a dearth of sensitive and reliable screening tests for most solid tumors. Here again, mouse models on inbred backgrounds with controllable and reproducible disease, coupled with advances in proteomic and molecular imaging technologies, may allow new diagnostic markers to be identified.[86]

Identification of the Cell of Origin

Spatial and temporal restriction of genetic alterations in mice also allow the initial events that are triggered by oncogene expression to be investigated and the cells that respond to these initial genetic lesions to be identified. Specific genetic manipulation in defined cell types can identify the cell type in a given tissue that is susceptible to oncogenic transformation.[87,88] Alternatively, analyzing the cells that respond after in vivo oncogene activation may identify the cells of origin.[89] The appeal of these approaches is not solely to identify tumor-initiating cells but also to allow their subsequent manipulation and the identification of critical pathways dysregulated in these cells.

Recruitment and Function of Immune, Vascular, and Stromal Cells in the Tumor Environment

It has become increasingly clear that tumor growth and progression are greatly influenced by surrounding nontumor cells, including various immune cell types, vascular cells, stromal fibroblasts, and myofibroblasts.[90,91] Mouse models in which each of these tumor cell populations can be manipulated independently allow the function of each cell type to be identified.[92] Moreover, molecules that regulate the recruitment, survival, and function of these cells within the tumor can be characterized in mouse models in vivo. The secreted and cell-surface molecules used by these cells to communicate with each other and with the tumor cells will lead to the identification of important regulators of tumor growth, angiogenesis, invasion, and metastasis.

Conclusions

Genetically engineered mouse models of human cancers are an important component of the arsenal of experimental systems that will allow the in vivo dissection of tumor biology over the next several decades. The versatility of mouse models that recapitulate human cancer will lead to timely identification and validation of therapeutic targets that will ultimately influence human health.

Key References

6. Hirst GL, Balmain A. Forty years of cancer modelling in the mouse. *Eur J Cancer*. 2004;40:1974-1980.
7. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov*. 2006;5:741-754.
8. Singh M, Johnson L. Using genetically engineered mouse models of cancer to aid drug development: an industry perspective. *Clin Cancer Res*. 2006;12:5312-5328.
9. Berghmans S, Jette C, Langenau D, et al. Making waves in cancer research: new models in the zebrafish. *Biotechniques*. 2005;39:227-237.
10. Poulin G, Nandakumar R, Ahringer J. Genome-wide RNAi screens in *Caenorhabditis elegans*: impact on cancer research. *Oncogene*. 2004;23:8340-8345.
11. Vidal M, Cagan RL. *Drosophila* models for cancer research. *Curr Opin Genet Dev*. 2006;16:10-16.
12. Pagliarini RA, Xu T. A genetic screen in *Drosophila* for metastatic behavior. *Science*. 2003;302:1227-1231.
17. Dickins RA, McJunkin K, Hernando E, et al. Tissue-specific and reversible RNA interference in transgenic mice. *Nat Genet*. 2007;39:914-921.
18. Premsrirut PK, Dow LE, Kim SY, et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell*. 2011;145:145-158.
23. Ji H, Li D, Chen L, et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell*. 2006;9:485-495.

25. Shachaf CM, Kopelman AM, Arvanitis C, et al. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature*. 2004;431:1112-1117.
27. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev*. 2006;20:1496-1510.
31. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature*. 2006;443:214-217.
33. Finch A, Prescott J, Shchors K, et al. Bcl-xL gain of function and p19 ARF loss of function cooperate oncogenically with Myc in vivo by distinct mechanisms. *Cancer Cell*. 2006;10:113-120.
34. Capecchi MR. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat Rev Genet*. 2005;6:507-512.
43. Olive KP, Tuveson DA, Ruhe ZC, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell*. 2004;119:847-860.
45. Forster A, Pannell R, Drynan LF, et al. Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. *Cancer Cell*. 2003;3:449-458.
46. Drynan LF, Pannell R, Forster A, et al. Mll fusions generated by Cre- loxP-mediated de novo translocations can induce lineage reassignment in tumorigenesis. *EMBO J*. 2005;24:3136-3146.
49. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T. Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med*. 2005;11:63-70.
50. Meuwissen R, Linn SC, Linnoila RI, Zevenhoven J, Mooi WJ, Berns A. Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. *Cancer Cell*. 2003;4:181-189.
51. DuPage M, Dooley AL, Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc*. 2009;4:1064-1072.
54. Lu J, Getz G, Miska EA, et al. Micro RNA expression profiles classify human cancers. *Nature*. 2005;435:834-838.
55. Kim M, Gans JD, Nogueira C, et al. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell*. 2006;125:1269-1281.
56. Zender L, Spector MS, Xue W, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell*. 2006;125:1253-1267.
57. Sweet-Cordero A, Mukherjee S, Subramanian A, et al. An oncogenic KRAS2 expression signature identified by cross-species gene-expression analysis. *Nat Genet*. 2005;37:48-55.
58. Winslow MM, Dayton TL, Verhaak RG, et al. Suppression of lung adenocarcinoma progression by Nkx2-1. *Nature*. 2011;473:101-104.
59. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
60. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
61. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
62. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 2003;21:4342-4349.
65. Batlle E, Bacani J, Begthel H, et al. EphB receptor activity suppresses colorectal cancer progression. *Nature*. 2005;435:1126-1130.
66. Ma L, Teruya-Feldstein J, Behrendt N, et al. Genetic analysis of Pten and Tsc2 functional interactions in the mouse reveals asymmetrical haploinsufficiency in tumor suppression. *Genes Dev*. 2005;19:1779-1786.
67. Chen ML, Xu PZ, Peng XD, et al. The deficiency of Akt1 is sufficient to suppress tumor development in Pten mice. *Genes Dev*. 2006;20:1569-1574.
68. Weng AP, Millholland JM, Yashiro-Ohtani Y, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev*. 2006;20:2096-2109.
71. Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell*. 2005;122:473-483.
72. Collier LS, Carlson CM, Ravimohan S, Dupuy AJ, Largaespada DA. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*. 2005;436:272-276.
73. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. *Nature*. 2005;436:221-226.
74. Kolfschoten IG, van Leeuwen B, Berns K, et al. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell*. 2005;121:849-858.
75. Westbrook TF, Martin ES, Schlabach MR, et al. A genetic screen for candidate tumor suppressors identifies REST. *Cell*. 2005;121:837-848.
76. Ngo VN, Davis RE, Lamy L, et al. A loss-of-function RNA interference screen for molecular targets in cancer. *Nature*. 2006;441:106-110.
80. Bric A, Miething C, Bialucha CU, et al. Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model. *Cancer Cell*. 2009;16:324-335.
81. Meacham CE, Ho EE, Dubrovsky E, Gertler FB, Hemann MT. In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. *Nat Genet*. 2009;41:1133-1137.
82. Zender L, Xue W, Zuber J, et al. An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. *Cell*. 2008;135:852-864.
83. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324:1457-1461.
84. Singh M, Lima A, Molina R, et al. Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. *Nat Biotechnol*. 2010;28:585-593.
85. Chen Z, Cheng K, Walton Z, et al. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature*. 2012;483:613-617.
86. Taguchi A, Politi K, Pitteri SJ, et al. Lung cancer signatures in plasma based on proteome profiling of mouse tumor models. *Cancer Cell*. 2011;20:289-299.
87. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. *Cell*. 2004;119:431-443.
88. Sutherland KD, Proost N, Brouns I, Adriaensen D, Song JY, Berns A. Cell of origin of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. *Cancer Cell*. 2011;19:754-764.
89. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*. 2005;121:823-835.
91. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432:332-337.
92. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*. 2010;330:827-830.
99. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469-483.
100. Jackson EL, Olive KP, Tuveson DA, et al. The differential effects of mutant p53 alleles on advanced murine lung cancer. *Cancer Res*. 2005;65:10280-10288.
101. Lu SL, Herrington H, Reh D, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev*. 2006;20:1331-1342.

This page intentionally left blank

References

1. Linzer DI, Levine AJ. Characterization of a 54K Dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*. 1979;17:43-52.
2. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature*. 1979;278:261-263.
3. Sheiness D, Bishop JM. DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. *J Virol*. 1979;31:514-521.
4. Stehelin D, Varmus HE, Bishop JM, et al. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature*. 1976;260:170-173.
5. Van Dyke T, Jacks T. Cancer modeling in the modern era: progress and challenges. *Cell*. 2002;108:135-144.
6. Hirst GL, Balmain A. Forty years of cancer modelling in the mouse. *Eur J Cancer*. 2004;40:1974-1980.
7. Sharpless NE, Depinho RA. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov*. 2006;5:741-754.
8. Singh M, Johnson L. Using genetically engineered mouse models of cancer to aid drug development: an industry perspective. *Clin Cancer Res*. 2006;12:5312-5328.
9. Berghmans S, Jette C, Langenau D, et al. Making waves in cancer research: new models in the zebrafish. *Biotechniques*. 2005;39:227-237.
10. Poulin G, Nandakumar R, Ahringer J. Genome-wide RNAi screens in *Caenorhabditis elegans*: impact on cancer research. *Oncogene*. 2004;23:8340-8345.
11. Vidal M, Cagan RL. *Drosophila* models for cancer research. *Curr Opin Genet Dev*. 2006;16:10-16.
12. Pagliarini RA, Xu T. A genetic screen in *Drosophila* for metastatic behavior. *Science*. 2003;302:1227-1231.
13. Adams JM, Harris AW, Pinkert CA, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature*. 1985;318:533-538.
14. Hanahan D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature*. 1985;315:115-122.
15. Brinster RL, Chen HY, Messing A, et al. Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. *Cell*. 1984;37:367-379.
16. Holland EC, Hively WP, DePinho RA, Varmus HE. A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice. *Genes Dev*. 1998;12:3675-3685.
17. Dickins RA, McJunkin K, Hernando E, et al. Tissue-specific and reversible RNA interference in transgenic mice. *Nat Genet*. 2007;39:914-921.
18. Premsrirut PK, Dow LE, Kim SY, et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell*. 2011;145:145-158.
19. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A*. 1992;89:5547-5551.
20. Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science*. 1995;268:1766-1769.
21. Furth PA, St Onge L, Böger H, et al. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci U S A*. 1994;91:9302-9306.
22. Fisher GH, Wellen SL, Klimstra D, et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev*. 2001;15:3249-3262.
23. Ji H, Li D, Chen L, et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell*. 2006;9:485-495.
24. Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol Cell*. 1999;4:199-207.
25. Shachaf CM, Kopelman AM, Arvanitis C, et al. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature*. 2004;431:1112-1117.
26. Chin L, Tam A, Pomerantz J, et al. Essential role for oncogenic Ras in tumour maintenance. *Nature*. 1999;400:468-472.
27. Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W, Varmus HE. Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev*. 2006;20:1496-1510.
28. Moody SE, Sarkisian CJ, Hahn KT, et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell*. 2002;2:451-461.
29. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res*. 1995;23:1686-1690.
30. Indra AK, Warot X, Brocard J, et al. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res*. 1999;27:4324-4327.
31. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature*. 2006;443:214-217.
32. Pelengaris S, Khan M, Evan GI. Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell*. 2002;109:321-334.
33. Finch A, Prescott J, Shchors K, et al. Bcl-xL gain of function and p19 ARF loss of function cooperate oncogenically with Myc in vivo by distinct mechanisms. *Cancer Cell*. 2006;10:113-120.
34. Capecchi MR. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat Rev Genet*. 2005;6:507-512.
35. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. Effects of an Rb mutation in the mouse. *Nature*. 1992;359:295-300.
36. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat Genet*. 1994;7:353-361.
37. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*. 1992;356:215-221.
38. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*. 1996;85:27-37.
39. Wallace MR, Marchuk DA, Andersen LB, et al. Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science*. 1990;249:181-186.
40. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*. 1990;250:1233-1238.
41. Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature*. 1990;348:747-749.
42. Groden J, Thliveris A, Samowitz W, et al. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*. 1991;66:589-600.
43. Olive KP, Tuveson DA, Ruhe ZC, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell*. 2004;119:847-860.
44. Gu H, Marth JD, Orban PC, Mossman H, Rajewsky K. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science*. 1994;265:103-106.
45. Forster A, Pannell R, Drynan LF, et al. Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. *Cancer Cell*. 2003;3:449-458.
46. Drynan LF, Pannell R, Forster A, et al. Mll fusions generated by Cre- loxP-mediated de novo translocations can induce lineage reassignment in tumorigenesis. *EMBO J*. 2005;24:3136-3146.
47. Jackson EL, Willis N, Mercer K, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev*. 2001;15:3243-3248.
48. Kalamarides M, Niwa-Kawakita M, Leblois H, et al. Nf2 gene inactivation in arachnoidal cells is rate-limiting for meningioma development in the mouse. *Genes Dev*. 2002;16:1060-1065.
49. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T. Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med*. 2005;11:63-70.

50. Meuwissen R, Linn SC, Linnoila RI, Zevenhoven J, Mooi WJ, Berns A. Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. *Cancer Cell*. 2003;4:181-189.
51. DuPage M, Dooley AL, Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc*. 2009;4:1064-1072.
52. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature*. 2000;408:307-310.
53. Barker CM, Calvert RJ, Walker CW, et al. Detection of mutant p53 in clam leukemia cells. *Exp Cell Res*. 1997;232:240-245.
54. Lu J, Getz G, Miska EA, et al. Micro RNA expression profiles classify human cancers. *Nature*. 2005;435:834-838.
55. Kim M, Gans JD, Nogueira C, et al. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell*. 2006;125:1269-1281.
56. Zender L, Spector MS, Xue W, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell*. 2006;125:1253-1267.
57. Sweet-Cordero A, Mukherjee S, Subramanian A, et al. An oncogenic KRAS2 expression signature identified by cross-species gene-expression analysis. *Nat Genet*. 2005;37:48-55.
58. Winslow MM, Dayton TL, Verhaak RG, et al. Suppression of lung adenocarcinoma progression by Nkx2-1. *Nature*. 2011;473:101-104.
59. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
60. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
61. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
62. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 2003;21:4342-4349.
63. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-1037.
64. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*. 1983;304:596-602.
65. Batlle E, Bacani J, Begthel H, et al. EphB receptor activity suppresses colorectal cancer progression. *Nature*. 2005;435:1126-1130.
66. Ma L, Teruya-Feldstein J, Behrendt N, et al. Genetic analysis of Pten andTsc2 functional interactions in the mouse reveals asymmetrical haploinsufficiency in tumor suppression. *Genes Dev*. 2005;19:1779-1786.
67. Chen ML, Xu PZ, Peng XD, et al. The deficiency of Aktl is sufficient to suppress tumor development in Pten mice. *Genes Dev*. 2006;20:1569-1574.
68. Weng AP, Millholland JM, Yashiro-Ohtani Y, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev*. 2006;20:2096-2109.
69. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev*. 1999;13:2678-2690.
70. Mikkers H, Allen J, Knipscheer P, et al. High-throughput retroviral tagging to identify components of specific signaling pathways in cancer. *Nat Genet*. 2002;32:153-159.
71. Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell*. 2005;122:473-483.
72. Collier LS, Carlson CM, Ravimohan S, Dupuy AJ, Largaespada DA. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. *Nature*. 2005;436:272-276.
73. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. *Nature*. 2005;436:221-226.
74. Kolfschoten IG, van Leeuwen B, Berns K, et al. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. *Cell*. 2005;121:849-858.
75. Westbrook TF, Martin ES, Schlabach MR, et al. A genetic screen for candidate tumor suppressors identifies REST. *Cell*. 2005;121:837-848.
76. Ngo VN, Davis RE, Lamy L, et al. A loss-of-function RNA interference screen for molecular targets in cancer. *Nature*. 2006;441:106-110.
77. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. *Cell*. 1991;65:753-763.
78. van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell*. 1991;65:737-752.
79. Suzuki T, Shen H, Akagi K, et al. New genes involved in cancer identified by retroviral tagging. *Nat Genet*. 2002;32:166-174.
80. Bric A, Miething C, Bialucha CU, et al. Functional identification of tumor-suppressor genes through an in vivo RNA interference screen in a mouse lymphoma model. *Cancer Cell*. 2009;16:324-335.
81. Meacham CE, Ho EE, Dubrovsky E, Gertler FB, Hemann MT. In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. *Nat Genet*. 2009;41:1133-1137.
82. Zender L, Xue W, Zuber J, et al. An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. *Cell*. 2008;135:852-864.
83. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324:1457-1461.
84. Singh M, Lima A, Molina R, et al. Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. *Nat Biotechnol*. 2010;28:585-593.
85. Chen Z, Cheng K, Walton Z, et al. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature*. 2012;483:613-617.
86. Taguchi A, Politi K, Pitteri SJ, et al. Lung cancer signatures in plasma based on proteome profiling of mouse tumor models. *Cancer Cell*. 2011;20:289-299.
87. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. *Cell*. 2004;119:431-443.
88. Sutherland KD, Proost N, Brouns I, Adriaensen D, Song JY, Berns A. Cell of origin of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. *Cancer Cell*. 2011;19:754-764.
89. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*. 2005;121:823-835.
90. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86:353-364.
91. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432:332-337.
92. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*. 2010;330:827-830.
93. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*. 1988;54:105-115.
94. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell*. 1989;57:931-936.
95. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A*. 1995;92:3439-3443.
96. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science*. 1992;256:668-670.
97. Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C, Taketo M. Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc Natl Acad Sci U S A*. 1995;92:4482-4486.
98. Fodde R, Edelmann W, Yang K, et al. A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A*. 1994;91:8969-8973.
99. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469-483.
100. Jackson EL, Olive KP, Tuveson DA, et al. The differential effects of mutant p53 alleles on advanced murine lung cancer. *Cancer Res*. 2005;65:10280-10288.
101. Lu SL, Herrington H, Reh D, et al. Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev*. 2006;20:1331-1342.

Hasan Korkaya, April Davis, and Max S. Wicha*

10

Cancer Stem Cells and the Microenvironment

Accumulating evidence suggests that most if not all tumors are maintained by a subpopulation of cells that display stem cell properties including self-renewal and lineage differentiation. Cancer stem cells (CSC) have been isolated from a number of human malignancies by using cell surface markers and enzymatic activity of cytoplasmic proteins. Subsequent characterization of these CSCs in mouse xenograft models revealed that these cells can mediate metastasis and contribute to treatment resistance and relapse. Furthermore, recent studies suggest that the CSCs are regulated by the components of tumor microenvironment through complex networks of cytokines and growth factors. Importantly, these components have a direct influence on CSC properties and thus may represent attractive targets for development of novel therapeutics. This chapter highlights advances in elucidating the networks between CSC and the tumor microenvironment and efforts to target these CSC regulatory networks.

Identification of Cancer Stem Cells

Embryonic and tissue-specific stem cells display two distinct properties: (1) self-renewal, or the ability of the cell to undergo several symmetric or asymmetric divisions while maintaining an undifferentiated cell pool; and (2) differentiation, or the ability to generate distinct cell types. Tissue-specific stem cells are distinguished from embryonic stem cells in that their differentiation is primarily limited to cell types of a particular organ. Tissue-specific stem cells have the capacity to self-renew as well as to differentiate into committed progenitors and terminally differentiated cells with specialized functions. There is increasing evidence that a similar hierarchy governs many human malignancies, including tumors of the hematopoietic system and solid organs. CSCs are operationally defined by their ability to initiate tumors in mice on serial passage, a demonstration of self-renewal, as well as their ability to differentiate into the non–self-renewing cells forming the tumor bulk.[1-3] Human CSC assays have used immunocompromised mice whereas mouse CSCs have also been identified via transplantation studies in syngeneic hosts.[4,5] In fact, these early studies on normal organ development and tumors suggested that the tumors are indeed organ-like structures resembling their normal counterparts in that they are both comprised of heterogeneous cell populations.

According to the CSC hypothesis, tumors are hierarchically organized whereby self-renewing CSCs drive tumorigenesis while differentiated cells form the tumor bulk.[6] The CSC model fundamentally differs from the traditional or "stochastic" model of carcinogenesis in which any cell may have equal malignant potential. Based on the stochastic model, most therapeutic strategies have been selected for their ability to cause tumor shrinkage by targeting rapidly cycling cells, whereas the CSC model predicts that targeting and elimination of self-renewing cancer stem cells will be necessary to significantly improve outcome and ultimately cure patients with cancer.

Despite the fact that the heterogeneity of tumor cells has been widely acknowledged, the CSC model was difficult to validate until appropriate mouse models were developed. The development of biomarkers to identify CSCs, as well as validation of in vitro and mouse models, has facilitated the isolation and characterization of these cells from both murine and human tumors. Bonnet and Dick were the first to describe the hierarchical organization of acute myeloid leukemia (AML) and the existence of CSCs[7] in that disease. The team isolated such cells from AML and demonstrated that a small subset of cells characterized by $CD34^+/CD38^-$ phenotype that made up less than 0.01% of tumor cells could transfer human AML into NOD/SCID mice, whereas the remaining cells that lacked this phenotype failed to do so.[7] Using similar techniques, CSCs have subsequently been isolated from many human

*Disclaimer: Max S. Wicha has financial holdings and is a scientific adviser for OncoMed Pharmaceuticals, is a scientific adviser for Veristem, and has research support from Dompe.

malignancies, including those of the brain, breast, colon, prostate, pancreas, lung, and liver.[3,8-14]

Examples of Cancer Stem Cells

Uchida and colleagues described a phenotype, $CD133^+$ $CD34^-CD45^-$, that is characterized by the expression of a cell surface antigen—Prominin-1 (CD133), a five-transmembrane glycoprotein—and lack of CD34 and CD45 to isolate human central nervous system stem cells.[15] Brain tumors displayed a cellular hierarchy reminiscent of their normal counterparts, in which $CD133^+$ tumor cells but not $CD133^-$ cells were able to form tumors in NOD-SCID mouse brains and neurospheres in in vitro cultures.[15]

A subpopulation of breast cancer cells that displayed stem cell properties was characterized by expression of the cell surface markers ESA (EpCam) and CD44 in the absence of CD24 expression.[3] As few as 200 $EpCam^+CD44^+/CD24^-Lin^-$ cells were able to generate tumors in immunocompromised NOD/SCID mice, whereas 100-fold more cells that did not express these markers isolated from the same tumors were nontumorigenic.[3] Furthermore, the tumor-initiating populations regenerated tumors that recapitulated the heterogeneity of the initial tumor.[3] Subsequently, both normal and malignant mammary stem cells were isolated by virtue of their increased expression of aldehyde dehydrogenase (ALDH), which can be accessed by Aldefluor assay.[2] Interestingly, $CD44^+/CD24^-$ and $ALDH^+$ identify overlapping but not identical cell populations.[2,16] Furthermore, these markers can be used to isolate CSC populations from established breast cancer cell lines, as well as primary tumor xenografts.[2,17]

In the normal prostate, $CD133^+$ cells have been shown to display properties of stem cells,[18] whereas the $CD44^+/\alpha2\beta1^{hi}/CD133^+$ phenotype (about 0.1% of the total tumor cells) marked prostate cancer stem cells.[19] As few as 500 cells with this phenotype were able to form tumors in mice, whereas 5×10^5 cells that lacked this phenotype failed to generate tumors.

Distinct pulmonary epithelial cells located at the bronchioalveolar ductal junction displayed functional stem cell properties in mouse models.[20] These cells, characterized by the $Sca1^+/CD34^+/CD45^-/Pecam^-$ phenotype, were able to generate lung epithelium after tissue damage, suggesting that these cells possess self-renewal and lineage differentiation properties. Furthermore, in K-Ras–induced mouse lung adenocarcinoma, a $Sca1^+/CD34^+/CD45^-/Pecam^-$ phenotype identified lung cancer stem cells capable of initiating tumors when transplanted into syngeneic mice.[20] In addition, human lung cancers contain a rare population of cells that express CD133[8] and ALDH1.[21] These cells are able to initiate tumors as well as to generate differentiated cells in mouse xenografts.

Tumor-initiating cells have also been isolated in colorectal tumors. Ricci-Vitiani and co-workers and O'Brien and colleagues, isolated $CD133^+$ human colon cancer cells and injected them subcutaneously or under the renal capsule of NOD-SCID mice.[12,13] Both groups demonstrated that $CD133^+$ cells not only were capable of forming tumors, but also generated tumors that recapitulated the cellular heterogeneity of the initiated tumors. In contrast, $CD133^-$ cells lacked these properties, suggesting that the capabilities of self-renewal and lineage differentiation are required for tumor initiation as well as generating non–self-renewing cells that constitute the tumor bulk.

Human pancreatic carcinomas contain a subpopulation of cells with a $CD44^+/CD24^+/ESA^+$ phenotype (0.2% to 0.8%) that were 100-fold more tumorigenic than those that did not express these markers.[11] These pancreatic CSCs also displayed activation of developmental pathways such as sonic hedgehog.

Human liver cancer stem cells characterized by the expression of CD133 demonstrated self-renewal potential, increased colony-forming ability, and tumor formation in mouse xenografts.[14] In addition, liver tumor cells that express CD90 (Thy1), glycosylphosphatidylinositol-anchored glycoprotein, and CD44 have been shown to be able to initiate tumors that are capable of metastasis in immunodeficient mice.[23]

The Origins of Cancer Stem Cells

The use of transplantation models to identify and characterize tumor-initiating cell populations has been criticized because these studies involve introducing cells into an environment that may differ from that found in de novo tumor initiation. However, several recent studies have used in situ lineage tracing directly to address this concern. These studies have demonstrated the generation of CSCs from tissue stem cells in mouse models of skin, gut, and brain.[24-26] Expression of the Wnt target Lgr5 marks intestinal stem cells and by using lineage tracing Schepers et al. demonstrated that Lgr5 expression also identifies a subpopulation (5-10%) of growing intestinal adenomas in mice. These $Lgr5^+$ cells self-renew to generate additional $Lgr5^+$ cells as well as other $Lgr5^-$ lineages.[24] Driessens and associates employed a similar genetic lineage tracing approach to mark individual skin papilloma cells and traced lineages derived from these cells at different stages of tumor progression in mice. Interestingly, a majority of labeled cells displayed limited proliferative potential, whereas a fraction of tumor cells were long lived and gave rise to progeny that formed the tumor bulk.[25]

Patients with glioblastoma have a median survival of about 1 year because of therapeutic resistance and recurrence after tumor resection. In order to understand the nature of cells that are responsible for relapse, Chen and colleagues used a genetically engineered mouse model of glioblastoma to demonstrate that repopulating tumor cells were nestin-positive cancer stem cells located in the subventricular zone.[26] These nestin-expressing glioblastoma cells were resistant to chemotherapy and repopulated the tumor after therapy. Together, these studies provide strong support for the cancer stem cell model by demonstrating cellular hierarchies in tumors that develop in situ without the requirement for transplantation.

Regulation of Cancer Stem Cells by the Tumor Microenvironment and Cytokine Networks

Although the preponderance of cancer research has focused on genetic alterations that initiate and drive cancer, there is substantial evidence that malignant transformation and tumor progression are also highly influenced by the tumor microenvironment. This research has suggested that tumors are in effect "organ-like structures" composed of different cell types whose interaction is required to drive and promote tumor growth and metastasis. In normal tissues, a small number of stem cells have the capacity to self-renew as well as to differentiate into multiple lineages. A similar hierarchy governs many human malignancies, including the tumors of the hematopoietic system and solid organs. Analogous to the regulation of normal stem cells by their "niche," CSCs are regulated by, and in turn, regulate, cells within the tumor microenvironment. Cells recruited to the microenvironment, including mesenchymal stem cells (MSCs), tissue-associated fibroblasts, and endothelial cells, interact with CSCs via growth factor and cytokine networks (Figure 10-1). Furthermore, immunomodulatory cells, including T cells and macrophages, exert both inhibitory and stimulatory effects on CSCs and their progeny. In addition, chronic inflammation characterized by increased generation of inflammatory cytokines such as IL-1, IL-6, and IL-8 may also regulate CSCs during carcinogenesis.[27-29] The interaction between CSCs and their microenvironment provides new targets for therapeutic intervention.

Emerging evidence also implicates CSCs in tumor metastasis and therapeutic resistance,[30-33] and therefore understanding the pathways that regulate this cell population is of clinical importance. As is the case with their

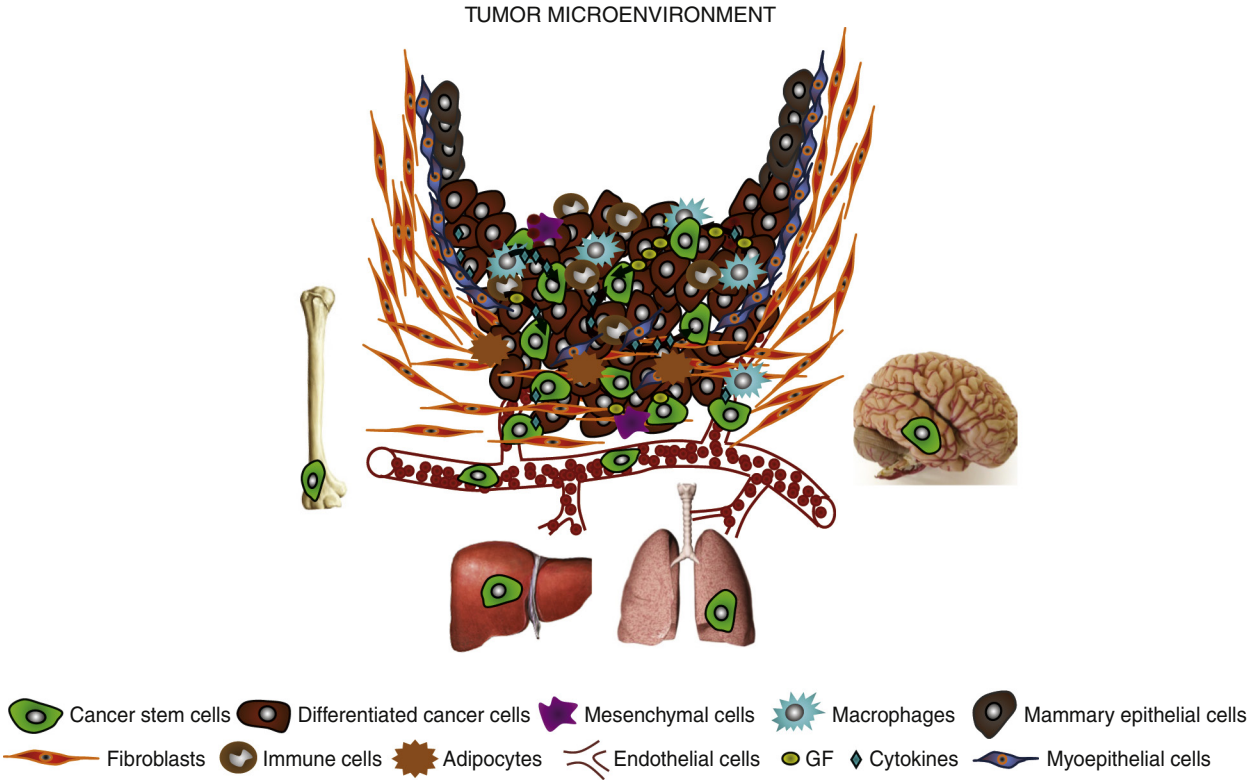


Figure 10-1 The tumor microenvironment Elevated levels of cytokines/growth factors produced by tumor cells enhance the proliferation and survival of cancer stem cells, induce angiogenesis, and recruit tumor-associated macrophages, neutrophils, and mast cells, which secrete additional growth factors, forming a positive feedback loop that promotes tumor cell invasion and distant metastasis.

normal counterparts, CSCs are regulated by intrinsic as well as extrinsic signals originating in the tumor microenvironment.[34] Epigenetic and genetic changes that occur during carcinogenesis modulate these regulatory signals within the tumor microenvironment.[35,36] Stem cell regulatory pathways frequently dysregulated in tumors include the Notch, Hedgehog, Wnt, PI-3K, NFκB, and Jak/STAT pathways.[16,37-40] Although these pathways are activated in some tumors via mutation of key regulatory elements, they are more often activated by signals from the tumor microenvironment.[41,42] Bi-directional paracrine signaling networks coordinately regulate tumorigenic cell populations, including cancer stem cells.[37,43-45] Tumor cells in turn produce factors that attract and regulate the diverse variety of cell types constituting the tumor microenvironment.[43,45] Interestingly, many of the pathways activated during tumor formation resemble those that mediate normal wound healing, including cytokine loops that in turn are regulated by the transcription factor NFκB.[37] In addition, immunomodulatory cells may manifest stimulatory or inhibitory effects on tumor progression.[46,47] Diverse cell populations within the tumor microenvironment may impinge on these pathways to regulate the CSC population. Elucidation of these networks is of clinical importance because it may allow for the identification of novel therapeutic targets and the development of strategies to target CSC populations.

Cellular Components of the Tumor Microenvironment That Influence Cancer Stem Cells

During tumor progression, tumor cells recruit a diverse collection of cells that make up the microenvironment, and through iterative interactions both the tumor cell and its microenvironment co-evolve.[42] For example, because of increased oxidative stress in the prostate tumor microenvironment, stromal cells acquire the ability to mimic other cell types such as mesenchymal and vascular stem cells.[48] Although early studies suggested that some of the cells in the tumor microenvironment harbored mutations,[22] more recent evidence suggests that mutations are limited to the tumorigenic cells, which through paracrine interactions modify the epigenetic program of nontumorigenic cells in the tumor microenvironment.[22,41,49,50] The cells in the microenvironment in turn interact with and generate epigenetic changes in tumor cells.[41,42] This reciprocal interaction is illustrated by changes in the tumor microenvironment that occur during the evolution of preinvasive ductal carcinoma in situ to invasive carcinoma of the breast, which involves sequential epigenetic changes in both the tumor as well as the stromal microenvironment.[41,42] Human bone stromal cells were able to induce aggressive mouse prostate tumors that acquired an EMT phenotype associated with resistance to radiation therapy.[51]

In human breast cancers, mesenchymal cells are recruited from the bone marrow[43] or from the normal breast stroma.[45] As is the case in breast cancer cells, ALDH expression is elevated in MSCs that are recruited from the bone marrow. These MSCs interact with breast CSCs through cytokine loops involving IL6 and CXCL7.[43] These cytokine loops stimulate the self-renewal of breast CSCs.[43] Immunohistochemical analysis has confirmed the existence of such MSC–breast CSC interactions in biopsies obtained from breast cancer patients.[43] In addition, MSCs have the ability to differentiate into adipocytes as well as tumor-associated fibroblasts that also interact with and influence tumor cells.[52]

The activation of fibroblasts and myofibroblasts was originally described in a study of wound healing by Gabbiani and Majno, who observed morphological changes in activated myofibroblasts compared to quiescent tumor- and wound-associated fibroblasts.[53] Based on the similarities between the wound healing process and cancer, both of which involve infiltration of inflammatory cells and activation of cytokine networks, it was proposed that malignant tumor cells are "wounds that don't heal."[54] It appears that the persistence of a wound healing environment promotes the persistence and/or expansion of CSCs. In an experimental mouse model, acute wounding in the mammary gland by dermal incision accelerated breast tumor growth and metastasis.[55] Although the exact mechanisms remain unknown, paracrine signals from evolving tumors induce epigenetic changes in the surrounding stromal fibroblasts.[56] Indeed, the gene expression profile of tumor-associated fibroblasts resembles that of wound-activated fibroblasts, and this profile is associated with poor prognosis.[57,58] Growth factors such as TGF-β may be involved in these epigenetic changes, leading to activation of fibroblasts.[59] In addition, cytokines such as SDF-1 (aka CXCL-12) produced by breast carcinoma–associated fibroblasts (but not normal fibroblasts) may promote the proliferation of tumor cells, which express the SDF-1 receptor CXCR4.[60] The level of expression of SDF-1 in serum has been associated with poor survival in breast cancer patients.[61,62] Other growth factors such as hepatocyte growth factor (HGF), produced by mammary stromal cells, may also have a profound effect on developing mammary tumors, possibly inducing the stem cell compartment.[63] HGF provides a co-stimulatory signal to the Wnt pathway during colon carcinogenesis.[64] Other important growth factors produced by activated fibroblasts include the fibroblast growth factors (FGFs). Cancer stem cells can use oxidative stress to drive stromal fibroblasts to produce necessary nutrients for their survival.[65] It has recently been shown that estrogen regulates

the breast CSC population through a paracrine mechanism involving FGF9.[66] Additional factors produced by stromal cells in the tumor microenvironment activate a number of pathways including IGF, PDGF, Wnt, NFκB, Notch, Hedgehog, and matrix metalloproteinases (MMPs) regulating tumor proliferation, invasion, and metastasis.[67-72] These pathways also have been implicated in regulating CSCs in a number of human malignancies.[37,40,73-76]

Immunomodulatory cells may also exert inhibitory and stimulatory effects on CSCs, and the ultimate balance of these effects may profoundly influence tumor growth/progression. The importance of the immune system is illustrated by recent studies elucidating the mechanisms by which macrophages recognize and destroy CSCs. Recent studies in human leukemia and lymphoma have suggested that tumor cells express the antigen CD47, which serves as a "don't eat me" signal to tumor-associated macrophages.[77] At the same time, these cells express calreticulin, recognized by these macrophages as an "eat me" signal.[78] Administration of a blocking antibody to CD47 induced macrophage phagocytosis of tumor cells in vitro and in mouse models. Importantly, it was demonstrated that leukemic stem cells as well as bulk tumor cells could be targeted by this approach.[78,79] Interestingly, CD47 is widely expressed in a number of solid tumor CSCs, and targeting of this molecule suppresses tumor growth and metastasis in mouse tumor models.[80]

Endothelial cells may also play an important role in the tumor microenvironment by directly interacting with tumor cells as well as by their role in blood vessel formation. Endothelial cells constitute an important component of normal hematopoietic and neuronal stem cell niches.[81,82] In addition, cytokines produced by endothelial cells directly regulate cancer stem cells.[83,84] More than 40 years ago, Judah Folkman proposed that angiogenesis, the process of new blood vessel formation, was required for tumor growth and metastasis.[85] The role of tumor angiogenesis has been demonstrated in preclinical models of many cancers, including those of the breast.[86] This has led to the development of a number of anti-angiogenic agents as cancer therapeutics. Angiogenesis is a complex process involving interactions among multiple cell types. Bone marrow–derived endothelial progenitor cells are attracted to tumors, where they differentiate into mature endothelial cells and capillaries.[87,88] These newly formed blood vessels carry oxygen and nutrients to growing tumors, facilitating progression and metastasis (see Figure 10-1). Interestingly, the tumor vasculature is vastly different from the normal vasculature, as illustrated by the finding that more than 1000 genes are differentially expressed, including FGFRs, MMPs, and JAK3.[83] Although preneoplastic lesions lack angiogenic capacity, transition from hyperplasia to neoplasia requires induction of angiogenesis, a process that may be regulated by NFκB.[89,90]

Tumors may also generate a vasculature by a process termed *vasculogenic mimicry* in which CSCs transdifferentiate into vessel-forming cells that resemble endothelium. Recent reports have demonstrated that glioblastoma CSCs are multipotent and can differentiate into endothelial cells, generating their own vasculature.[91,92] Although many pro-angiogenic factors have been identified, vascular endothelial growth factor (VEGF) is the primary mediator of this process,[93] and as a result it has been the principal target of anti-angiogenic therapies. A humanized monoclonal antibody targeting VEGF, bevacizumab, as well as two multikinase VEGF inhibitors, sorafenib and sunitinib, are currently approved for clinical use. These anti-angiogenic agents have shown utility in a number of tumor types, including renal and colon cancers.[94,95]

Bevacizumab was initially approved for use in metastatic breast cancer on the basis of reports demonstrating that it prolonged time to tumor progression.[96] However, more recent studies (AVADO and RIBBON1) have suggested that this effect is limited and that the addition of bevacizumab to cytotoxic chemotherapy failed to increase patient survival.[97] These results are consistent with reports in mouse models that anti-angiogenic agents may accelerate local breast cancer invasion and distant metastasis.[98,99] In mice bearing human breast cancer xenografts, these agents increase the CSC population through the generation of tissue hypoxia[100] and activation of HIF1α-Wnt signaling. Anti-angiogenic agents may also stimulate CSC expansion by increasing HGF production by tumor stromal cells.[101] These studies provide a rationale for the addition of CSC targeting agents to anti-angiogenic agents to improve clinical efficacy.

Cytokine Networks Can Promote Cancer Stem Cell Self-Renewal

The link between inflammation and cancer is an old concept that was first proposed by Virchow in 1864, when he observed that inflammatory cells frequently infiltrate the tumor stroma.[102] Considerable clinical evidence suggests links between inflammatory states and cancer development, including the well-documented association of ulcerative colitis, hepatitis C, and chronic pancreatitis with cancers of the colon, liver, and pancreas, respectively.[102] States of chronic inflammation as assessed by serum C reactive protein or β amyloid are correlated with risk of breast cancer recurrence in women after primary therapy.[103] This chronic inflammatory state may be mediated by cytokines including IL-1β, IL-6, and IL-8.[27] Genetic polymorphisms in these cytokine genes predispose affected individuals to cancer.[104] These inflammatory cytokines stimulate CSC self-renewal, which then may promote tumor growth and metastasis.[44,105]

IL-6 and IL-8 have been implicated both in chronic inflammation and in tumor growth.[106,107] Within the tumor microenvironment, many cell types, including mesenchymal cells, macrophages, and immune cells, secrete both IL-6 and IL-8.[106] In patients with advanced breast cancer, the serum levels of both of these cytokines have been associated with poor patient outcome.[108,109] In a variety of preclinical models, IL-6 has been shown to promote tumorigenicity, angiogenesis, and metastasis.[44,110-112] IL-6 has been shown to be a direct regulator of breast CSC self-renewal, a process mediated by the IL-6 receptor/GP130 complex through activation of STAT3.[37] Using mouse xenografts, we have recently demonstrated that bone marrow–derived MSCs are recruited to sites of growing breast cancers by gradients of IL-6.[43] IL-6 is a key component of a positive feedback loop involving these MSCs and CSCs.[43] Furthermore, Sethi and colleagues recently demonstrated that IL-6–mediated Jagged1/Notch signaling promotes breast cancer bone metastasis.[73] These studies identify IL-6 and its receptor as attractive therapeutic targets to deplete CSCs.

Using gene expression profiling we previously demonstrated that the IL-8 receptor CXCR1 is highly expressed on breast CSCs[105]. Interestingly, cytotoxic chemotherapy induced cell death in differentiated cancer cells that resulted in increased production of IL-8, which in turn stimulated breast CSCs via activating CXCR1. A small-molecule CXCR1 inhibitor, reparaxin, significantly reduced CSC in breast cancer xenografts, inhibiting tumor growth and metastasis. Based on these preclinical studies, a clinical trial using reparaxin combined with established chemotherapy has been initiated.

The production of inflammatory cytokines including IL-6 and IL-8 is regulated by the NFκB signaling pathway.[113] The NFκB pathway plays a crucial role in inflammation and carcinogenesis. The NFκB family is composed of five related transcription factors: p50, p52, RelA (p65), c-Rel, and RelB.[114,115] In resting cells, NFκB proteins are predominantly located in the cytoplasm where they associate with the IκB family of proteins; activation of NFκB by diverse signals results in ubiquitin ligase–dependent degradation of IκB, which results in nuclear translocation of NFκB protein complexes. The transcription of a number of cytokines, including IL-6 and IL-8, is activated by NFκB.[113] In addition, a positive feedback loop maintains a chronic inflammatory state in tumor cells. Interestingly, this loop involves the microRNA let7, as well as Lin28, a factor involved in embryonic stem cell self-renewal.[37] This feedback loop is maintained by IL-6 through its activation of Stat3, which in turn activates NFκB and its downstream targets Lin28 and let7. The specific role of IL-6 in maintaining this inflammatory loop in BCSCs has been recently demonstrated.[37,40] NFκB may play an important role in normal breast physiology as well as carcinogenesis. In a HER2/neu mouse model of mammary carcinogenesis, suppression of NFκB in mammary epithelium reduced the mammary stem cell compartment, resulting in a delayed generation of HER2-neu–induced tumors, which displayed reduced angiogenesis and infiltration by macrophages.[90] NFκB has also been implicated in the regulation of mouse mammary stem cells during pregnancy. Elevated levels of progesterone during pregnancy induce the production of RANK ligand (RANKL) by differentiated breast epithelial cells. RANKL in turn stimulates breast stem cell self-renewal via activation of NFκB in these cells.[116,117] The increased incidence of aggressive breast cancers associated with pregnancy[118] may result from activation of these pathways in breast CSCs.[116,117]

Epidemiologic studies demonstrate that obesity is associated with a significant increase in postmenopausal breast cancer,[119,120] which may be related to the known link between obesity and inflammation.[121-124] Patients with type 2 diabetes mellitus (T2DM) have elevated levels of circulating proinflammatory cytokines including TNF-α, IL-6, and C-reactive protein.[125] In addition, these elevated levels of proinflammatory cytokines have been linked to NFκB activation.[126] The diabetes drug metformin reduces levels of circulating glucose, increases insulin sensitivity, and reduces insulin resistance-associated hyperinsulinemia. Interestingly, in preclinical mouse models, metformin has been shown to selectively inhibit CSC self-renewal, reducing the proliferation of these cells in breast tumors.[127]

Summary

As organ-like structures, tumors are composed of diverse cellular compartments and networks that play important roles in tumor progression. Accumulating evidence suggests that these cellular components and cytokine networks also regulate CSCs, which in turn drive tumor growth and metastasis. One can reframe Paget's "seed and soil" hypothesis of tumor metastasis[128] in a modern context. The "seeds" are the cancer stem cells, and the "soil" is the rich microenvironment composed of diverse cell types that interact with tumor cells via growth factor and cytokine networks (see Figure 10-1). These networks regulate CSCs and their progeny, which propagate CSC as well as generating non–self-renewing cells that constitute the tumor bulk. Studies in preclinical models have demonstrated that targeting of regulatory pathways such as IL-6, IL-8, and NFκB can effectively reduce the CSC populations in breast cancer as well as other tumor types.

Key References

1. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene*. 2008;27:6120-6130.
2. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*. 2007;1:555-567.
4. Zhang M, Behbod F, Atkinson RL, et al. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer Res*. 2008;68:4674-4682.
5. Duarte S, Loubat A, Momier D, et al. Isolation of head and neck squamous carcinoma cancer stem-like cells in a syngeneic mouse model and analysis of hypoxia effect. *Oncol Rep*. 2012;28:1057-1062.
6. O'Brien CA, Kreso A, Jamieson CH. Cancer stem cells and self-renewal. *Clin Cancer Res*. 2010;16:3113-3120.
8. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. 2008;15:504-514.
9. Ding W, Mouzaki M, You H, et al. CD133$^+$ liver cancer stem cells from methionine adenosyl transferase 1A-deficient mice demonstrate resistance to transforming growth factor (TGF)-beta-induced apoptosis. *Hepatology*. 2009;49:1277-1286.
11. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res*. 2007;67:1030-1037.
12. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2007;445:106-110.
13. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature*. 2007;445:111-115.
14. Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology*. 2007;132:2542-2556.
16. Korkaya H, Paulson A, Charafe-Jauffret E, et al. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol*. 2009; 7: e1000121.
17. Charafe-Jauffret E, Ginestier C, Iovino F, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res*. 2009;69:1302-1313.
18. Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci*. 2004;117:3539-3545.
19. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*. 2005;65:10946-10951.
20. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*. 2005;121:823-835.
21. Ucar D, Cogle CR, Zucali JR, et al. Aldehyde dehydrogenase activity as a functional marker for lung cancer. *Chem Biol Interact*. 2009;178:48-55.
22. Patocs A, Zhang L, Xu Y, et al. Breast-cancer stromal cells with TP53 mutations and nodal metastases. *N Engl J Med*. 2007;357:2543-2551.
23. Yang ZF, Ho DW, Ng MN, et al. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell*. 2008;13:153-166.
24. Schepers AG, Snippert HJ, Stange DE, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science*. 2012;337:730-735.
25. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. *Nature*. 2012;488:527-530.
26. Chen J, Li Y, Yu TS, et al. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature*. 2012;488:522-526.
28. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev*. 2011;22:83-89.
29. Mantovani A, Pierotti MA. Cancer and inflammation: a complex relationship. *Cancer Lett*. 2008;267:180-181.
30. Liu H, Patel MR, Prescher JA, et al. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci U S A*. 2010;107:18115-18120.
31. Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*. 2008;26:2839-2845.
32. Eyler CE, Foo WC, LaFiura KM, McLendon RE, Hjelmeland AB, Rich JN. Brain cancer stem cells display preferential sensitivity to Akt inhibition. *Stem Cells*. 2008;26:3027-3036.
33. Li X, Lewis MT, Huang J, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst*. 2008;100:672-679.
37. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell*. 2009;139:693-706.
38. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res*. 2006;66:6063-6071.
39. Dontu G, Jackson KW, McNicholas E, Kawamura J, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res*. 2004;6:R605-R615.
41. Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res*. 2009;11:R7.
42. Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. *Trends Genet*. 2009;25:30-38.
43. Liu S, Ginestier C, Ou SJ, et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res*. 2011;71:614-624.
44. Sansone P, Storci G, Tavolari S. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest*. 2007;117:3988-4002.
45. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
46. Mantovani A. Cancer: inflaming metastasis. *Nature*. 2009;457:36-37.
47. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer*. 2009;9:798-809.
48. Josson S, Matsuoka Y, Chung LW, Zhau HE, Wang R. Tumor-stroma co-evolution in prostate cancer progression and metastasis. *Semin Cell Dev Biol*. 2010;21:26-32.
49. Trimboli AJ, Cantemir-Stone CZ, Li F, et al. Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*. 2009;461:1084-1091.
50. Campbell IG, Qiu W, Polyak K, Haviv I. Breast-cancer stromal cells with TP53 mutations. *N Engl J Med*. 2008;358:1634-1635; author reply 1636.
51. Josson S, Sharp S, Sung SY, et al. Tumor-stromal interactions influence radiation sensitivity in epithelial- versus mesenchymal-like prostate cancer cells. *J Oncol*. 2010.
55. Stuelten CH, Barbul A, Busch JI, et al. Acute wounds accelerate tumorigenesis by a T cell-dependent mechanism. *Cancer Res*. 2008;68:7278-7282.
56. Hu M, Yao J, Cai L, et al. Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet*. 2005;37:899-905.
57. Farmer P, Bonnefoi H, Anderle P, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med*. 2009;15:68-74.
58. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008;14:518-527.
59. Bierie B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer*. 2006;6:506-520.
60. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.
61. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood*. 2006;107:1761-1767.
63. Michieli P, Mazzone M, Basilico C, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell*. 2004;6:61-73.
65. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: a new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. *Cell Cycle*. 2010;9:3256-3276.
67. Dufraine J, Funahashi Y, Kitajewski J. Notch signaling regulates tumor angiogenesis by diverse mechanisms. *Oncogene*. 2008;27:5132-5137.
73. Sethi N, Dai X, Winter CG, Kan Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell*. 2011;19:192-205.
74. Du Z, Li J, Wang L, et al. Overexpression of DeltaNp63alpha induces a stem cell phenotype in MCF7 breast carcinoma cell line through the Notch pathway. *Cancer Sci*. 2010;101:2417-2424.

75. Rizzo P, Osipo C, Pannuti A, Golde T, Osborne B, Miele L. Targeting Notch signaling cross-talk with estrogen receptor and ErbB-2 in breast cancer. *Adv Enzyme Regul*. 2009;49:134-141.
76. Medina V, Calvo MB, Diaz-Prado S, Espada J. Hedgehog signalling as a target in cancer stem cells. *Clin Transl Oncol*. 2009;11:199-207.
77. Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142:699-713.
78. Chao MP, Jaiswal S, Weissman-Tsukamoto R, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med*. 2010;2:63-94.
79. Chao MP, Alizadeh AA, Tang C, et al. Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. *Cancer Res*. 2011;71:1374-1384.
80. Willingham SB, Volkmer JP, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A*. 2012;109:6662-6667.
81. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. 2007;11:69-82.
82. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 2006;25:977-988.
83. Bhati R, Patterson C, Livasy CA, et al. Molecular characterization of human breast tumor vascular cells. *Am J Pathol*. 2008;172:1381-1390.
84. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell*. 2007;129:465-472.
86. Huh JI, Calvo A, Stafford J, et al. Inhibition of VEGF receptors significantly impairs mammary cancer growth in C3(1)/Tag transgenic mice through antiangiogenic and non-antiangiogenic mechanisms. *Oncogene*. 2005;24:790-800.
87. Duda DG, Cohen KS, Kozin SV, et al. Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. *Blood*. 2006;107:2774-2776.
94. Okines A, Cunningham D. Current perspective: bevacizumab in colorectal cancer—a time for reappraisal? *Eur J Cancer*. 2009;45:2452-2461.
96. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med*. 2007;357:2666-2676.
97. Petrelli F, Barni S. Bevacizumab in advanced breast cancer: an opportunity as second-line therapy? *Med Oncol*. 2012;29(1):226-230.
98. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 2009;15:220-231.
99. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell*. 2009;15:232-239.
100. Santos SJKP, Heath A, Clouthier S, Wicha M. Anti-angiogenic agents increase breast cancer stem cells via generation of tumor hypoxia. *AACR Annu Meeting*. 2011:3336.
103. Pierce BL, Ballard-Barbash R, Bernstein L, et al. Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. *J Clin Oncol*. 2009;27:3437-3444.
104. Michaud DS, Daugherty SE, Berndt SI, et al. Genetic polymorphisms of interleukin-1B (IL-1B), IL-6, IL-8, and IL-10 and risk of prostate cancer. *Cancer Res*. 2006;66:4525-4530.
105. Ginestier C, Liu S, Diebel ME, et al. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest*. 2010;120:485-497.
106. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. 2008;14:6735-6741.
107. Scheller J, Rose-John S. Interleukin-6 and its receptor: from bench to bedside. *Med Microbiol Immunol*. 2006;195:173-183.
108. Yao C, Lin Y, Chua MS, et al. Interleukin-8 modulates growth and invasiveness of estrogen receptor-negative breast cancer cells. *Int J Cancer*. 2007;121:1949-1957.
109. Benoy IH, Salgado R, Van Dam P, et al. Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clin Cancer Res*. 2004;10:7157-7162.
110. Gao SP, Mark KG, Leslie K, et al. Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J Clin Invest*. 2007;117:3846-3856.
112. Ara T, Declerck YA. Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer*. 2010;46:1223-1231.
114. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene*. 2006;25:6706-6716.
115. Moynagh PN. The NF-kappaB pathway. *J Cell Sci*. 2005;118:4589-4592.
119. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol*. 2008;9:367-377.
120. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. *J Pathol*. 2003;201:221-228.
122. Basen-Engquist K, Chang M. Obesity and cancer risk: recent review and evidence. *Curr Oncol Rep*. 2011;13:71-76.
123. Jee SH, Kim HJ, Lee J. Obesity, insulin resistance and cancer risk. *Yonsei Med J*. 2005;46:449-455.
125. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest*. 2005;115:1111-1119.
127. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res*. 2009;69:7507-7511.

References

1. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene*. 2008;27:6120-6130.
2. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*. 2007;1:555-567.
3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;100:3983-3988.
4. Zhang M, Behbod F, Atkinson RL, et al. Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer Res*. 2008;68:4674-4682.
5. Duarte S, Loubat A, Momier D, et al. Isolation of head and neck squamous carcinoma cancer stem-like cells in a syngeneic mouse model and analysis of hypoxia effect. *Oncol Rep*. 2012;28:1057-1062.
6. O'Brien CA, Kreso A, Jamieson CH. Cancer stem cells and self-renewal. *Clin Cancer Res*. 2010;16:3113-3120.
7. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730-737.
8. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ*. 2008;15:504-514.
9. Ding W, Mouzaki M, You H, et al. CD133+ liver cancer stem cells from methionine adenosyl transferase 1A-deficient mice demonstrate resistance to transforming growth factor (TGF)-beta-induced apoptosis. *Hepatology*. 2009;49:1277-1286.
10. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res*. 2003;63:5821-5828.
11. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res*. 2007;67:1030-1037.
12. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2007;445:106-110.
13. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature*. 2007;445:111-115.
14. Ma S, Chan KW, Hu L, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology*. 2007;132:2542-2556.
15. Uchida N, Buck DW, He D, et al. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A*. 2000;97:14720-14725.
16. Korkaya H, Paulson A, Charafe-Jauffret E, et al. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol*. 2009;7: e1000121.
17. Charafe-Jauffret E, Ginestier C, Iovino F, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res*. 2009;69:1302-1313.
18. Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci*. 2004;117:3539-3545.
19. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*. 2005;65: 10946-10951.
20. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*. 2005;121:823-835.
21. Ucar D, Cogle CR, Zucali JR, et al. Aldehyde dehydrogenase activity as a functional marker for lung cancer. *Chem Biol Interact*. 2009;178:48-55.
22. Patocs A, Zhang L, Xu Y, et al. Breast-cancer stromal cells with TP53 mutations and nodal metastases. *N Engl J Med*. 2007;357:2543-2551.
23. Yang ZF, Ho DW, Ng MN, et al. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell*. 2008;13:153-166.
24. Schepers AG, Snippert HJ, Stange DE, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science*. 2012;337:730-735.
25. Driessens G, Beck B, Caauwe A, Simons BD, Blanpain C. Defining the mode of tumour growth by clonal analysis. *Nature*. 2012;488:527-530.
26. Chen J, Li Y, Yu TS, et al. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature*. 2012;488:522-526.
27. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860-867.
28. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev*. 2011;22:83-89.
29. Mantovani A, Pierotti MA. Cancer and inflammation: a complex relationship. *Cancer Lett*. 2008;267:180-181.
30. Liu H, Patel MR, Prescher JA, et al. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci U S A*. 2010;107:18115-18120.
31. Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*. 2008;26:2839-2845.
32. Eyler CE, Foo WC, LaFiura KM, McLendon RE, Hjelmeland AB, Rich JN. Brain cancer stem cells display preferential sensitivity to Akt inhibition. *Stem Cells*. 2008;26:3027-3036.
33. Li X, Lewis MT, Huang J, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst*. 2008;100:672-679.
34. Vera-Ramirez L, Sanchez-Rovira P, Ramirez-Tortosa CL, et al. Gene-expression profiles, tumor microenvironment, and cancer stem cells in breast cancer: latest advances towards an integrated approach. *Cancer Treat Rev*. 2010;36:477-484.
35. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet*. 2000;16:168-174.
36. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003;33(suppl):245-254.
37. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell*. 2009;139:693-706.
38. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res*. 2006;66:6063-6071.
39. Dontu G, Jackson KW, McNicholas E, Kawamura J, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res*. 2004;6:R605-R615.
40. Iliopoulo D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell*. 2010;39:493-506.
41. Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res*. 2009;11:R7.
42. Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. *Trends Genet*. 2009;25:30-38.
43. Liu S, Ginestier C, Ou SJ, et al. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res*. 2011;71:614-624.
44. Sansone P, Storci G, Tavolari S. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J Clin Invest*. 2007;117:3988-4002.
45. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
46. Mantovani A. Cancer: inflaming metastasis. *Nature*. 2009;457:36-37.
47. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer*. 2009;9:798-809.
48. Josson S, Matsuoka Y, Chung LW, Zhau HE, Wang R. Tumor-stroma co-evolution in prostate cancer progression and metastasis. *Semin Cell Dev Biol*. 2010;21:26-32.
49. Trimboli AJ, Cantemir-Stone CZ, Li F, et al. Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*. 2009;461:1084-1091.
50. Campbell IG, Qiu W, Polyak K, Haviv I. Breast-cancer stromal cells with TP53 mutations. *N Engl J Med*. 2008;358:1634-1635; author reply 1636.
51. Josson S, Sharp S, Sung SY, et al. Tumor-stromal interactions influence radiation sensitivity in epithelial- versus mesenchymal-like prostate cancer cells. *J Oncol*. 2010 doi.10.1155/2010/232831.
52. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
53. Gabbiani G, Majno G. Dupuytren's contracture: fibroblast contraction? An ultrastructural study. *Am J Pathol*. 1972;66:131-146.
54. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med*. 1986;315:1650-1659.
55. Stuelten CH, Barbul A, Busch JI, et al. Acute wounds accelerate tumorigenesis by a T cell-dependent mechanism. *Cancer Res*. 2008;68:7278-7282.
56. Hu M, Yao J, Cai L, et al. Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet*. 2005;37:899-905.

57. Farmer P, Bonnefoi H, Anderle P, et al. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med*. 2009;15:68-74.
58. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008;14:518-527.
59. Bierie B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer*. 2006;6:506-520.
60. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.
61. Burger JA, Kipps TJ. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood*. 2006;107:1761-1767.
62. Chu QD, Panu L, Holm NT, Li BD, Johnson LW, Zhang S. High chemokine receptor CXCR4 level in triple negative breast cancer specimens predicts poor clinical outcome. *J Surg Res*. 2010;159:689-695.
63. Michieli P, Mazzone M, Basilico C, et al. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell*. 2004;6:61-73.
64. Vermeulen L, De Sousa EMF, van der Heijden M, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol*. 2010;12:468-476.
65. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, et al. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co-evolution: a new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. *Cell Cycle*. 2010;9:3256-3276.
66. Fillmore CM, Gupta PB, Rudnick JA, et al. Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. Proc Natl Acad Sci U S A. 2010. 14;107(59)21737-21742.
67. Dufraine J, Funahashi Y, Kitajewski J. Notch signaling regulates tumor angiogenesis by diverse mechanisms. *Oncogene*. 2008;27:5132-5137.
68. Singer CF, Kronsteiner N, Marton E, et al. MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. *Breast Cancer Res Treat*. 2002;72:69-77.
69. Pietras K, Rubin K, Sjoblom T, et al. Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. *Cancer Res*. 2002;62:5476-5484.
70. LeBedis C, Chen K, Fallavollita L, Boutros T, Brodt P. Peripheral lymph node stromal cells can promote growth and tumorigenicity of breast carcinoma cells through the release of IGF-I and EGF. *Int J Cancer*. 2002;100:2-8.
71. Hiscox S, Parr C, Nakamura T, Matsumoto K, Mansel RE, Jiang WG. Inhibition of HGF/SF-induced breast cancer cell motility and invasion by the HGF/SF variant, NK4. *Breast Cancer Res Treat*. 2000;59:245-254.
72. Dale TC, Weber-Hall SJ, Smith K, et al. Compartment switching of WNT-2 expression in human breast tumors. *Cancer Res*. 1996;56:4320-4323.
73. Sethi N, Dai X, Winter CG, Kan Y. Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell*. 2011;19:192-205.
74. Du Z, Li J, Wang L, et al. Overexpression of DeltaNp63alpha induces a stem cell phenotype in MCF7 breast carcinoma cell line through the Notch pathway. *Cancer Sci*. 2010;101:2417-2424.
75. Rizzo P, Osipo C, Pannuti A, Golde T, Osborne B, Miele L. Targeting Notch signaling cross-talk with estrogen receptor and ErbB-2 in breast cancer. *Adv Enzyme Regul*. 2009;49:134-141.
76. Medina V, Calvo MB, Diaz-Prado S, Espada J. Hedgehog signalling as a target in cancer stem cells. *Clin Transl Oncol*. 2009;11:199-207.
77. Chao MP, Alizadeh AA, Tang C, et al. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell*. 2010;142:699-713.
78. Chao MP, Jaiswal S, Weissman-Tsukamoto R, et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Sci Transl Med*. 2010;2:63-94.
79. Chao MP, Alizadeh AA, Tang C, et al. Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. *Cancer Res*. 2011;71:1374-1384.
80. Willingham SB, Volkmer JP, Gentles AJ, et al. The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A*. 2012;109:6662-6667.
81. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. 2007;11:69-82.
82. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 2006;25:977-988.
83. Bhati R, Patterson C, Livasy CA, et al. Molecular characterization of human breast tumor vascular cells. *Am J Pathol*. 2008;172:1381-1390.
84. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell*. 2007;129:465-472.
85. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
86. Huh JI, Calvo A, Stafford J, et al. Inhibition of VEGF receptors significantly impairs mammary cancer growth in C3(1)/Tag transgenic mice through antiangiogenic and non-antiangiogenic mechanisms. *Oncogene*. 2005;24:790-800.
87. Duda DG, Cohen KS, Kozin SV, et al. Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. *Blood*. 2006;107:2774-2776.
88. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. 2001;7:1194-1201.
89. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature*. 1989;339:58-61.
90. Liu M, Sakamaki T, Casimiro MC, et al. The canonical NF-κB pathway governs mammary tumorigenesis in transgenic mice and tumor stem cell expansion. *Cancer Res*. 2010;70:10464-10473.
91. Wang R, Chadalavada K, Wilshire J, et al. Glioblastoma stem-like cells give rise to tumour endothelium. *Nature*. 2010;468:829-833.
92. Ricci-Vitiani L, Pallini R, Biffoni M, et al. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature*. 2010;468:824-828.
93. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene*. 1999;18:5356-5362.
94. Okines A, Cunningham D. Current perspective: bevacizumab in colorectal cancer—a time for reappraisal? *Eur J Cancer*. 2009;45:2452-2461.
95. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med*. 2003;349:427-434.
96. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med*. 2007;357:2666-2676.
97. Petrelli F, Barni S. Bevacizumab in advanced breast cancer: an opportunity as second-line therapy? *Med Oncol*. 2012;29(1):226-230.
98. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 2009;15:220-231.
99. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell*. 2009;15:232-239.
100. Santos SJKP, Heath A, Clouthier S, Wicha M. Anti-angiogenic agents increase breast cancer stem cells via generation of tumor hypoxia. *AACR Annu Meeting*. 2011:3336.
101. Shojaei F, Lee JH, Simmons BH, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer Res*. 2010;70:10090-10100.
102. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539-545.
103. Pierce BL, Ballard-Barbash R, Bernstein L, et al. Elevated biomarkers of inflammation are associated with reduced survival among breast cancer patients. *J Clin Oncol*. 2009;27:3437-3444.
104. Michaud DS, Daugherty SE, Berndt SI, et al. Genetic polymorphisms of interleukin-1B (IL-1B), IL-6, IL-8, and IL-10 and risk of prostate cancer. *Cancer Res*. 2006;66:4525-4530.
105. Ginestier C, Liu S, Diebel ME, et al. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest*. 2010;120:485-497.
106. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. *Clin Cancer Res*. 2008;14:6735-6741.
107. Scheller J, Rose-John S. Interleukin-6 and its receptor: from bench to bedside. *Med Microbiol Immunol*. 2006;195:173-183.
108. Yao C, Lin Y, Chua MS, et al. Interleukin-8 modulates growth and invasiveness of estrogen receptor-negative breast cancer cells. *Int J Cancer*. 2007;121:1949-1957.

109. Benoy IH, Salgado R, Van Dam P, et al. Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clin Cancer Res*. 2004;10:7157-7162.
110. Gao SP, Mark KG, Leslie K, et al. Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J Clin Invest*. 2007;117:3846-3856.
111. Conze D, Weiss L, Regen PS, et al. Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. *Cancer Res*. 2001;61:8851-8858.
112. Ara T, Declerck YA. Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer*. 2010;46:1223-1231.
113. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*. 1997;336:1066-1071.
114. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene*. 2006;25:6706-6716.
115. Moynagh PN. The NF-kappaB pathway. *J Cell Sci*. 2005;118:4589-4592.
116. Joshi PA, Jackson HW, Beristain AG, et al. Progesterone induces adult mammary stem cell expansion. *Nature*. 2010;465:803-807.
117. Asselin-Labat ML, Vaillant F, Sheridan JM, et al. Control of mammary stem cell function by steroid hormone signalling. *Nature*. 2010;465:798-802.
118. Peck JD, Hulka BS, Poole C, Savitz DA, Baird D, Richardson BE. Steroid hormone levels during pregnancy and incidence of maternal breast cancer. *Cancer Epidemiol Biomarkers Prev*. 2002;11:361-368.
119. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol*. 2008;9:367-377.
120. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. *J Pathol*. 2003;201:221-228.
121. Bennett NB, Ogston CM, McAndrew GM, Ogston D. Studies on the fibrinolytic enzyme system in obesity. *J Clin Pathol*. 1966;19:241-243.
122. Basen-Engquist K, Chang M. Obesity and cancer risk: recent review and evidence. *Curr Oncol Rep*. 2011;13:71-76.
123. Jee SH, Kim HJ, Lee J. Obesity, insulin resistance and cancer risk. *Yonsei Med J*. 2005;46:449-455.
124. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *Lancet Oncol*. 2002;3:565-574.
125. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest*. 2005;115:1111-1119.
126. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med*. 2005;11:183-190.
127. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res*. 2009;69:7507-7511.
128. Fidler IJ. The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. *Nat Rev Cancer*. 2003;3:453-458.

Yan Li, Olena Barbash, and J. Alan Diehl

11

Regulation of the Cell Cycle

Basic Principles of Cell Cycle Progression

The essential function of cell cycle control is the regulated duplication of the cells' genetic blueprint and the division of this genetic material such that one copy is provided to each daughter cell following division. The cell cycle can be divided conceptually into four individual phases. The "business" phases include *S phase* or synthesis phase, which is the period during which DNA is replicated, and mitosis (*M phase*), where DNA is packaged, the cells divide, and DNA is distributed to daughter cells. S phase and M phase are separated by Gap phases (G phase) to provide the cell with a proofreading period to ensure that DNA replication is completed and packaged appropriately prior to division. Separating M phase from S phase is the first Gap phase (G_1 phase) and separating S phase from M phase is the second Gap phase (G_2 phase). G_0 or *quiescence* occurs when cells exit the cell cycle because of the absence of growth-promoting signals or the presence of prodifferentiation signals. Ordered progression through each phase is intricately regulated through both positive and negative regulatory signaling molecules and is the basis of normal organismal development. The consequences of deregulated growth control include failed or altered development and/or neoplastic/cancerous growth. Over the past two decades, a detailed picture of the major regulators of cell cycle control in both model organisms and higher eukaryotes has evolved. In this chapter, we describe the major regulators of cell division control and introduce current concepts regarding their participation in cell growth.

The Cyclin-Dependent Kinases

Cell cycle progression is positively regulated by a family of protein kinases referred to as the *cyclin-dependent kinases* (CDKs). In yeast, the organism in which early groundbreaking work defined many major cell cycle regulators, a single CDK regulates cell division: CDC2 (*Schizosaccharomyces pombe*, fission yeast) and CDC28 (*Saccharomyces cerevisiae*, budding yeast). In contrast, multicellular organisms use a distinct CDK whose activity promotes transition through each cell cycle phase (Figure 11-1). CDKs are binary enzymes. The catalytic subunit, the CDK, coordinates adenosine triphosphate (ATP) and transfers phosphate to appropriate substrates. As a monomer, the CDK has no enzymatic activity; activation requires association with a specific allosteric activator called a *cyclin*. CDK subunits associate with specific cyclins (Table 11-1) during distinct phases of the cell cycle and, as active protein kinases, trigger transition through cell cycle phases. Although some CDKs can form complexes with multiple cyclins, in most cases active complexes rely on specific partnerships.

Homology among CDKs, at the level of primary amino acid sequence, is in the range of 30% to 40%. The most highly conserved sequence, which contributes directly to cyclin binding, is the PSTAIRE sequence (CDK1, CDK2) or PV/ISTVRE (CDK4, CDK6) where letters refer to individual amino acids comprising this sequence (e.g., P = proline).[1]

Cyclins associate with the CDK subunit through a conserved domain within the cyclin called the *cyclin box*. The crystal structure of cyclins has revealed that the cyclin box comprises two sets of five α helices that share little primary homology, but share significant homology with respect to structural and folding topology.[2] Sequences N- and C-terminal to the cyclin box share little if any homology and contribute to substrate-specific interactions and to posttranslational regulation of cyclin protein accumulation (e.g., protein degradation).

Posttranslational Regulation of CDKs

Regulation of CDKs by Phosphorylation

Cyclin binding to the CDK contributes to kinase activation by inducing a conformational change wherein a C-terminal

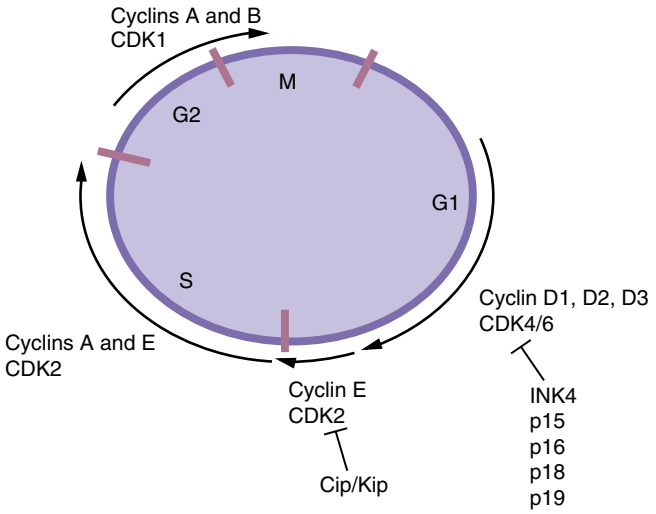


Figure 11-1 The cell cycle.

Table 11-1 CDKs, Activating Cyclins, and Select Substrates

CDK	Cyclin Partner	Substrate
CDKi (CDC_2)	A and B	Lamins, histone Hi
CDK_2	E and A	Rb, P107, P130, Cdt_1, CP110
CDK_3	C	Rb
CDK_4	D	Rb, P107, P130, $SMAD_2$, and $SMAD_3$
CDK_6	D	Rb, P107, P130, $SMAD_2$, and $SMAD_3$
CDK_7 (CAK)	H	CDK_1-CDK_6, RNA pol 11

CDK, Cyclin-dependent kinase.

domain of the CDK, referred to as the *T loop*, is directed out of the substrate binding cleft.[3] In the absence of cyclin binding, the T loop occludes substrate interactions. The cyclin-induced alteration, however, is not sufficient for complete CDK activation. T-loop displacement is ensured by direct phosphorylation of a conserved threonine residue within the T loop (Thr161, CDK1; Thr160, CDK2; Thr172, CDK4) by the CDK-activating kinase, CAK (Figure 11-2). In mammalian cells, CAK itself is a cyclin-dependent kinase composed of CDK7 and cyclin H.[4] CAK is constitutively active and contributes to CDK activation following cyclin binding via phosphorylation of the T-loop threonine.

Shortly after the identification of CDK7/cyclin H as CAK, CDK7/cyclin H was shown to be the previously identified activity referred to as *TFIIH*,[5] demonstrating that CAK (CDK7/cyclin H) not only contributes to CDK activation but is also implicated in transcriptional regulation. TFIIH phosphorylates multiple serine/threonine residues located in the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII), thereby contributing to increased transcriptional initiation.[5,6] CDK7 is also conserved in budding yeast. However, in yeast, CDK7 does not contribute to CDK activation. Rather, it is solely a

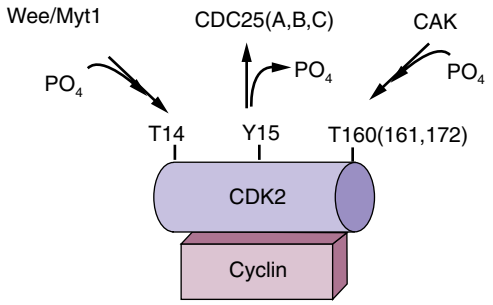


Figure 11-2 Regulation of cyclin-dependent kinase (CDK) CDKs are binary enzymes composed of a catalytic subunit, CDK, and a regulatory subunit, cyclin. Activation requires phosphorylation of a C-terminal threonine by the CDK-activating enzyme, CAK. In contrast, phosphorylation of N-terminal threonine and tyrosine residues inhibits adenosine triphosphate (ATP) binding and thus CDK activity.

regulator of RNA polymerase activity. Bona fide CAK activity in yeast is contributed by a distinct protein, CAK1.[7,8]

CDK phosphorylation is not solely an activating event. Phosphorylation of N-terminal threonine and tyrosine residues near the ATP binding pocket is inhibitory. Phosphorylation of threonine 14/tyrosine 15 is catalyzed by two enzymes, Wee1 and Myt1 (see Figure 11-2). Although Wee1 is a cytosolic enzyme, Myt1 is localized to endoplasmic reticulum structures.[9] The significance of the differential localization of Wee1 versus Myt1 remains to be established. Threonine 14/tyrosine 15 is located adjacent to the ATP binding pocket of CDKs, providing a structural basis for how phosphorylation of these residues prevents ATP binding.[10] Both threonine and tyrosine residues are conserved in CDK1-3, but only the tyrosine residue is present in CDK4-6. Although phosphorylation of CDK1-2 contributes to the timing of their activation during a normal cell cycle, the CDK4/6 enzymes appear to be subject to this inhibitory phosphorylation only when cells incur DNA damage.[11]

In mammalian cells, the removal of N-terminal inhibitory phosphates is catalyzed by any one of three highly related dual-specificity protein phosphatases: CDC25A, CDC25B, or CDC25C.[12] In contrast, yeast cells harbor a single CDC25 isoform that carries out all relevant functions. CDC25 isoforms are expressed in a cell cycle–dependent manner, and the A-B-C designation corresponds to their order of expression during the cell cycle. CDC25 A is expressed first, with its expression peaking at the G_1/S boundary. CDC25B expression follows that of CDC25A, with the highest levels detected during S phase. Finally, CDC25C is expressed during late G_2 and mitosis. From this expression pattern, substrate specificity was inferred, with CDC25A targeting the G_1 CDK_S (CDK4/6 or CDK2-cyclin E), CDC25B regulating the S-phase CDKs (CDK2-cyclin A), and CDC25C regulating mitotic CDKs (CDK1-cyclin B). Consistent with this hypothesis, inhibition of CDC25A resulted in increased CDK2-cyclin E tyrosine phosphorylation.[13] Also consistent

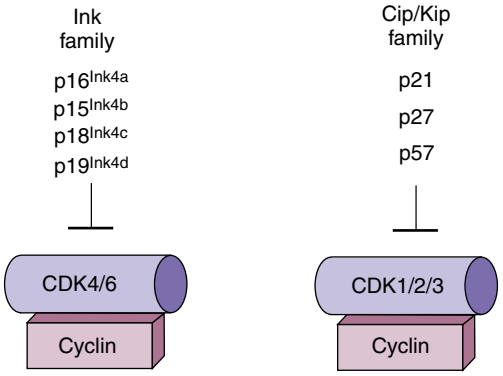


Figure 11-3 Regulation of cyclin-dependent kinases (CDKs) by polypeptide inhibitors Two distinct families of CDK inhibitors (CKIs) regulate CDK activity. The Cip/ Kip family binds with varying affinities to all CDK/cyclin complexes, but have the greatest inhibitory activity toward CDK2. The Ink4 family (inhibitor of CDK4/6) binds specifically to CDK4/6 and has no capacity to directly regulate other CDKs.

with substrate specificity being determined by the timing of expression, CDC25 enzymes do not exhibit any specificity toward distinct CDK substrates in vitro. However, timing of expression is not the sole determinant. Deletion of CDC25B or CDC25C, or even the combined deletion does not impair mouse development or cell proliferation in vitro.[14] It appears from this analysis that CDC25A expression is sufficient to drive cell cycle expression.

CDK Regulation by Small-Polypeptide Inhibitors

In addition to CDK regulation via phosphorylation, CDKs are subject to direct regulation by small-polypeptide inhibitory proteins referred to as *CDK inhibitors*, or CKIs (Figure 11-3).[15] These regulators bind directly to and inactivate CDK-cyclin complexes. There are two families of CKIs that have distinct biochemical activities. The *Ink4* (inhibitors of CDK4) family proteins bind exclusively to G_1 CDKs CDK4 and CDK6. Binding can directly inhibit an active CDK4/6-cyclin complex, or Ink4 protein can bind to monomeric CDK4/6 and prevent cyclin association. The second family, *Cip/Kip* family proteins, bind to a broad range of CDK-cyclin complexes but functionally appear to be negative regulators of CDK2 complexes.

Ink4 Family

The Ink4 family of proteins consists of four members: $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$. All four proteins bind exclusively to and inhibit D-type cyclin-dependent kinases CDK4 and CDK6. The founding member of the Ink4a family was discovered as a protein that interacted with CDK4 in co-immunoprecipitation experiments,[16] subsequently identified as MTS1.

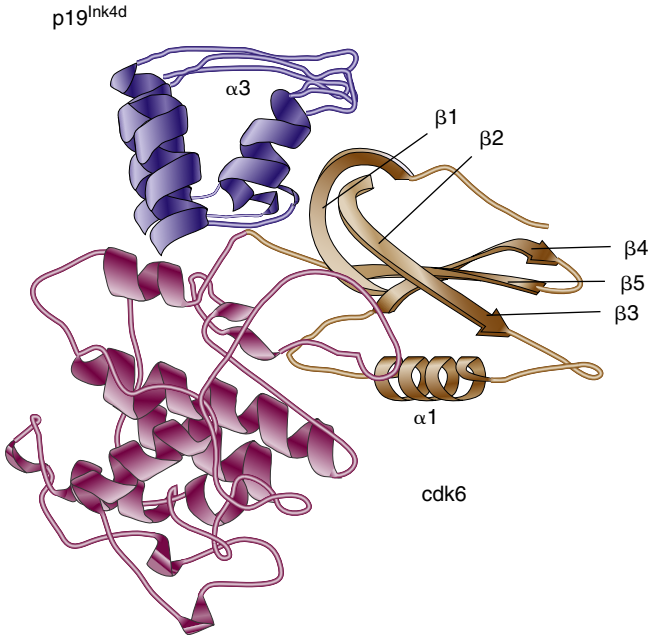


Figure 11-4 Three-dimensional structure of the $p19^{Ink4d}$/Cdk6 complex $p19^{Ink4d}$ is dark blue, apart from helix α3, which is light blue. The C-terminal domain of Cdk6 is dark brown, whereas the N-terminal domain, which undergoes extensive movement, is light brown.

Ink4 proteins are homologous in primary structure, sharing the presence of four or five ankyrin repeats, which are responsible for protein-protein interactions with CDK4/6. Each repeat consists of an extended strand connected by a *helix-loop-helix* (HLH) motif to the next extended strand. The crystal structure of the $p19^{Ink4d}$-CDK6 complex has been solved and provided valuable details about the mechanism of CDK inhibition by Ink proteins (Figure 11-4).[17] α-Helices and β-turns of $p19^{Ink4d}$ ankyrin repeats form a "cap" over the N-terminal domain of CDK6 and induce its spatial movement away from the C terminus. This event inhibits productive ATP binding but does not interfere with the formation of CDK-cyclin complex. As expected from their structure, all four Ink proteins exhibit similar biochemical activities toward CDK4 and CDK6. Interestingly, a short peptide that was derived from one of the ankyrin motifs had the ability to bind and inhibit CDK4, implying the importance of these domains in Ink4 functionality.[18]

Despite similar biochemical activities and comparable tertiary structures of Ink proteins, their regulation is distinct. $p16^{Ink4a}$ is not expressed in most tissues. Rather, it is induced in response to expression of oncogenic or transforming proteins and during cellular senescence. Several oncogenes as well as tumor suppressors regulate $p16^{Ink4a}$ expression. For example, overexpression of Ras increases $p16^{Ink4a}$ levels in primary rodent cells.[19] Inactivation of the retinoblastoma susceptibility protein, Rb, or tumor suppressor p53 can also promote $p16^{Ink4a}$ expression.[20] In contrast, $p15^{Ink4b}$ expression is regulated by growth-inhibitory factors

(anti-mitogens) such as TGF-β. Only $p18^{Ink4c}$ and $p19^{Ink4d}$ expression seems to be regulated during various phases of the cell cycle, with expression peaking during S phase.[21] The expression patterns of Ink4 proteins are also differentially regulated during development.

The structure of the genomic Ink4a locus is unique. Transcription through this locus gives rise to two biochemically distinct proteins, $p16^{Ink4a}$ and $p19^{ARF}$, as a result of alternative exon utilization.[22] Although $p16^{Ink4a}$ regulates CDK4/CDK6 activity, thereby indirectly regulating the Rb tumor suppressor, $p19^{ARF}$ regulates the p53 tumor suppressor.[23] $p19^{ARF}$ acts by attenuating Mdm2-mediated degradation of p53 and is known as an activator of the p53 pathway. Therefore, loss of $p19^{ARF}$ leads to impairment of p53 signaling. Elimination of the Ink4a/ARF genetic locus in mice makes the animals highly prone to tumor development.[24]

Cip/Kip Family

The Cip/Kip family of CKIs includes three members: $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. Unlike the Ink4 family of CKIs, Cip/Kip inhibitors bind to and efficiently inhibit various CDKs. Members of the Cip/Kip family are highly homologous and share approximately 50% of their sequences. The amino terminus of both $p21^{Cip1}$ and $p27^{Kip1}$ contains an RXL (where X is typically basic) sequence that is responsible for binding to cyclins and is called the *cyclin-binding motif*. Cip/Kip inhibitors also contain a domain that is responsible for the binding to CDKs (N-terminal in $p21^{Cip1}$ and $p27^{Kip1}$).[25] The crystal structure of the $p27^{Kip1}$/cyclin A/cdk2 complex (Figure 11-5) revealed that $p27^{Kip1}$ binds CDK2 at its N terminus and inserts into the catalytic cleft, thus mimicking ATP.[26] On cyclin A/CDK2, $p27^{Kip1}$ binds to the groove of the cyclin box. Because both Ink and Cip/Kip proteins occupy almost the same binding sites on CDKs, binding is mutually exclusive. For example, in vitro, $p15^{Ink4b}$ inhibits binding of $p27^{Kip1}$. However, in cells, which protein gets to the CDK first is often determined by the coordinated cellular localization of the inhibitors and cyclin-CDK complexes.

$p27^{Kip1}$ is responsible for induction and maintenance of the quiescent state. $p27^{Kip1}$ expression is induced in response to growth factor withdrawal and on contact inhibition in cell cultures.[27] $p27^{Kip1}$ levels are decreased on addition of the mitogens by various mechanisms described in subsequent paragraphs. Overexpression of $p27^{Kip1}$ in cells leads to cell cycle arrest in G_1 phase. Unlike $p27^{Kip1}$, $p21^{Cip1}$ is present at high levels in cycling cells, keeping CDK activities under tight control. $p21^{Cip1}$ levels are induced in response to DNA damage and genotoxic stress as a result of activation of p53. Similar to $p21^{Cip1}$ and $p27^{Kip1}$, induction of $p57^{Kip2}$ can mediate cell cycle arrest in G_1 phase. In addition, $p57^{Kip2}$ also participates in the M-to-G_1 transition through activation by p73.[28] Abrogation of p73 or its downstream effector $p57^{KIP2}$ perturbs mitotic progression and transition to G_1 phase.[29]

Transcriptional Regulation by the E2F Transcription Factors

E2F was originally identified as a cellular DNA binding activity that regulated expression of the viral E2 promoter.[30,31] Since this seminal work, molecular analysis has revealed that the E2F activity is encoded by a family of DNA binding proteins, which includes transcriptional activators and repressors. Mammalian cells encode eight known E2F proteins (E2F1-8; Figure 11-6). Further complication

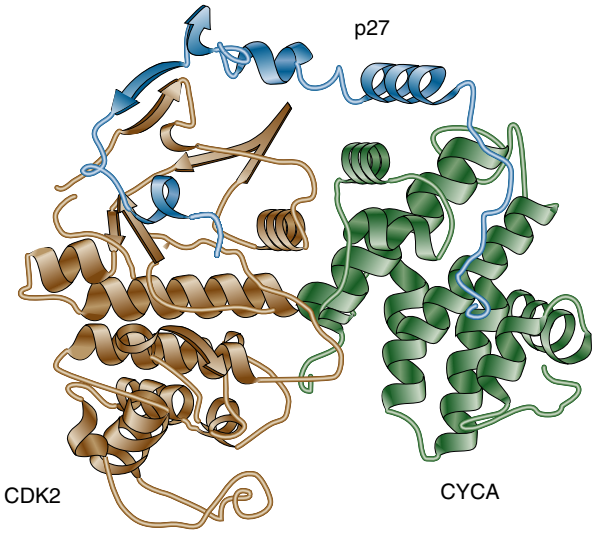


Figure 11-5 Cyclin A/CDK_2/$p27^{KIP1}$ complex Crystal structure of the inhibited ternary cyclin A/CDK_2/$p27^{Kip1}$ complex.

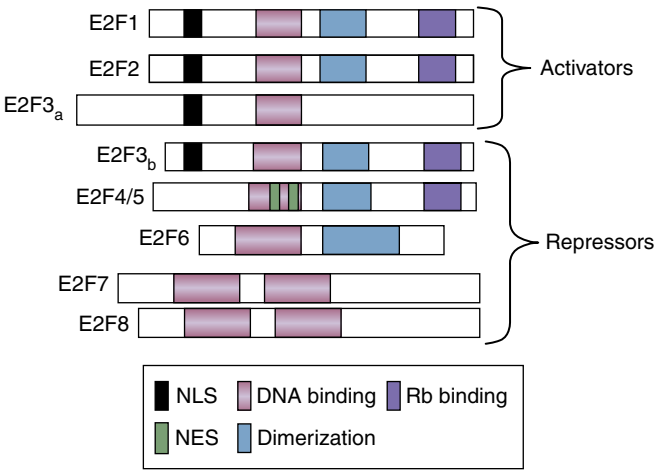


Figure 11-6 E2F family of transcription factors There are eight members of the E2F family of transcription factors. $E2F_5$ are classified as transcriptional activators or repressors. Functional domains are indicated by differential shading.

ensues from the fact that E2F associates with DNA as a heterodimer; the two known heterodimeric partners for E2F are DP1 and DP2. Indeed, the founding member, E2F1, can drive S-phase entry in the absence of growth factor stimulation.[32] The ability of E2F1 to drive S phase derives from its role in the regulation of genes whose protein products play essential roles in S-phase progression. Established E2F targets include components of DNA replication complexes (MCMs) and S-phase cyclins (E and A).[33] E2F family members were initially considered requisite regulators of S-phase entry. E2F1, E2F2, and E2F3 accumulate during G_1 phase and play critical roles in promoting expression of S-phase targets. Strikingly, E2F4 through E2F7 function as transcriptional repressors;[34] E2F3b, an alternatively spliced isoform of E2F3, is also a transcriptional repressor. The E2F repressors function to maintain cells in a quiescent or resting state. In addition to DP1, E2F complexes are further modulated by members of the retinoblastoma protein (pRb) family (pRb, p107, p130; Figure 11-7). The Rb family member functions to recruit chromatin-remodeling enzymes, such as histone deacetylases, to E2F complexes. As a consequence, increased activity of E2F1 through E2F3 requires dissociation of "pRb" from the E2F/DP1 heterodimer. As illustrated in the following sections, the G_1 CDK/cyclin kinase triggers this through direct phosphorylation of the associated pRb family member.[35]

In addition to the regulation of S-phase entry and progression, E2F transcriptional activators can trigger apoptosis or cell suicide. The mechanisms whereby E2F induces cell death remain unclear. However, pro-apoptotic genes have been identified as E2F target genes. Examples include the $p19^{ARF}$ protein, which is a known regulator of the p53 tumor suppressor. In addition, E2F can increase expression of pro-apoptotic proteins Puma, Noxa, and Bim and repress the anti-apoptotic Bcl2 family protein, Mcl1.

Two new E2F family members recently identified, E2F7 and E2F8, provide an important constraint against excessive E2F1 activation. Unlike the other mammalian E2Fs, E2F7 and E2F8 have two DNA-binding domains and do not require a DP partner to bind to DNA and as such are classified as atypical E2F family members.[36] These atypical E2Fs bind to the consensus E2F recognition sequence and can repress expression from a subgroup of cell cycle–regulated E2F targets. An E2F7 and E2F8 double knockout is an embryonic lethal resulting from massive apoptosis; this phenotype can be bypassed by removing E2F1 or p53.[37] Our current level of understanding underscores E2F7 and E2F8 as a distinct arm of the E2F network involved in repression of transcription during S-G_2 and control of the E2F1-p53 apoptotic axis.

G_1 Regulation/Restriction Point Control

During the first Gap phase or G_1, cells prepare for DNA replication. They must synthesize proteins necessary to replicate their genome, and once these are made, assemble the various components of the DNA replication machinery on chromatin at so-called origins of replication. This is

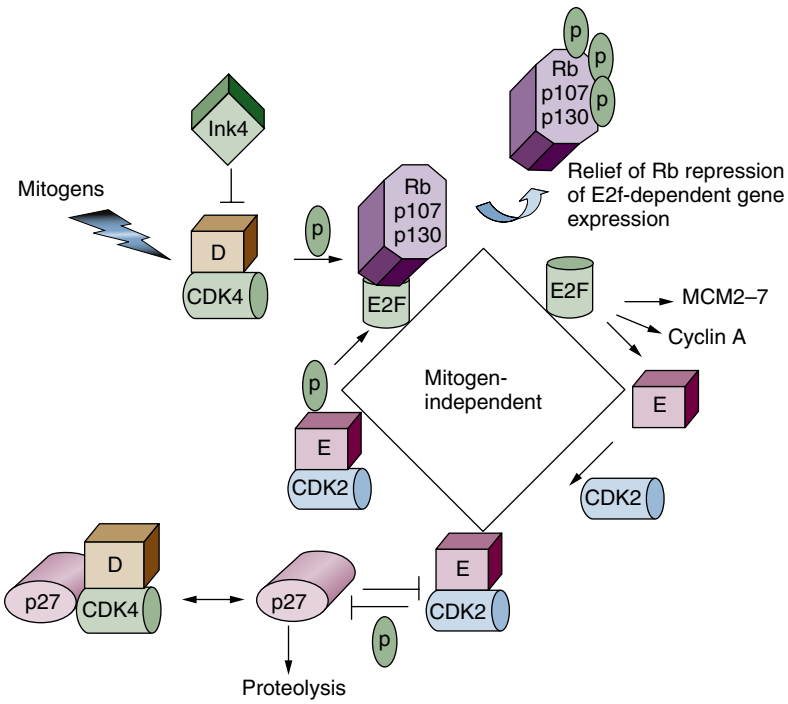


Figure 11-7 Restriction point control Progression through G_1 phase requires growth factor–mediated (mitogenic) signals. Mitogens promote the activation of the initial cyclin-dependent kinase (CDK; cyclin D/CDK4) complex, which phosphorylates Rb family proteins (inactivating signal). The CDK4 enzyme also binds to Cip/Kip CDK inhibitors (CKIs), thereby sequestering these proteins to facilitate CDK2 activation. Rb phosphorylation releases the transcription activating E2FS (E2F1-E2F3), which promote transcription of downstream targets such as cyclin E, A, and MCM proteins. Cyclin E binds to CDK2, and this active complex maintains Rb in an inactive state. Active cyclin E/CDK2 also targets its own inhibitor ($p27^{Kip1}$) for proteolysis via site-specific phosphorylation. The complete activation of CDK2, first by cyclin E and then by cyclin A, marks passage through the restriction point. Once past this point, cells no longer require growth factor stimulation for progression through the remainder of that cell division.

coordinated with nutrient and growth factor availability to ensure that the cell is in an environment that supports cell division. The G_1 phase of the cell cycle is unique in that it represents the only time wherein cells are sensitive to signals from their extracellular environment. These signals are in the form of adhesion to substratum and growth factors. Cells require growth factor–dependent signals up to a point in late G_1 referred to as the *restriction point* ("start" in yeast).

Progression through G_1 phase is driven by the collective activities of two distinct CDKs. The first is CDK4 or CDK6 in combination with a D-type cyclin. Mammalian cells encode three distinct D cyclins (D1, D2, D3), which are expressed in a tissue-specific manner. Whereas CDK4 and CDK6 are constitutively expressed, D cyclins are expressed in response to growth factor signaling. Following accumulation of active cyclin D/CDK4 or CDK6, the CDK2 kinase in combination with cyclin E accumulates to facilitate the transition from G_1 to S phase.

A key protein that regulates G_1-phase progression in the mammalian cell cycle is retinoblastoma protein, Rb. The Rb family consists of three members, Rb, p107, and p130. In quiescent cells, Rb proteins associate with E2F transcription factors to repress E2F-dependent transcription. E2F targets include genes responsible for regulation of cell cycle and DNA replication, such as cyclins E and A (see Figure 11-7). Rb activity is regulated at the level of posttranslational modification, specifically phosphorylation. Hypophosphorylated Rb is active and binds to E2F, thereby silencing E2F-dependent activity. Hypophosphorylated Rb family proteins therefore play a central role in maintaining cells in a resting or quiescent state. Quiescent cells reenter the cell cycle in response to mitogenic growth factors. Growth factor signaling induces the expression of D-type cyclins at transcriptional and posttranslational levels,[38] leading to activation of cyclin D–dependent kinases CDK4 and -6 and subsequent Rb phosphorylation. Cyclin D/CDK4 or -6 complexes also have a kinase-independent function. They sequester $p21^{Cip1}$ and $p27^{Kip1}$ CDK1s from CDK2 kinases and allow activation of basal CDK2/cyclin E kinases, which further phosphorylate Rb family proteins. Phosphorylation of Rb promotes its dissociation from E2F, allowing transcriptional activation of E2F targets such as cyclin E. The E2F-dependent spike in cyclin E, and thus CDK2/cyclin E activity, represents the transition from mitogen-dependent to mitogen-independent cell cycle progression (or passage through the restriction point). In addition to maintaining Rb proteins in a hyperphosphorylated (inactive) state, the activation of cyclin E/CDK2 promotes proteasome-dependent degradation of its own inhibitor $p27^{Kip1}$ (described in a subsequent section). These changes, which include cyclin D/CDK4/6 and cyclin E/CDK2 activation, Rb phosphorylation, and destruction of $p27^{Kip1}$, render cells with decreased mitogen dependency and are irreversibly committed to enter S phase of the cell cycle.

Regulation of DNA Replication (S Phase)

Early experimentation, which relied on techniques wherein two cells (generally one human and one rodent cell) in distinct phases of the cell cycle are fused together (one cytoplasm containing the two distinct nuclei), revealed that chromosomes were competent for duplication in G_1 and S phases. For example, fusing an S-phase cell with a G_1-phase cell could enforce replication of a G_1 cell; in contrast, fusion of a cell in G_2 phase with an S-phase cell could not enforce replication of G_2 chromosomes. It was inferred from these experiments that S-phase cells contained a factor that triggered replication initiation and that G_1 chromosomes were prepared or "licensed" for this initiating activity. Research efforts have shed light on the molecular basis of regulated replication initiation.

Although DNA is actively replicated during S phase, cells must prepare DNA for replication during the preceding G_1 phase. During G_1 phase, *origins* (chromatin positions where DNA polymerase complexes initiate replication) must first be established or "licensed." Licensing refers to the formation of the pre-RC (pre-replication complex) at origins of replication (Figure 11-8). Initially, the origin of replication complex (ORC) must be associated with chromatin to act as a landing pad on which the pre-RC is formed. Unlike most components of the pre-RC, ORC remains constitutively bound to DNA. In budding yeast, ORC acts as a sequence-specific DNA binding complex; however, in fission yeast and mammalian cells, no sequence specificity has been elucidated for ORC. The next step is the recruitment

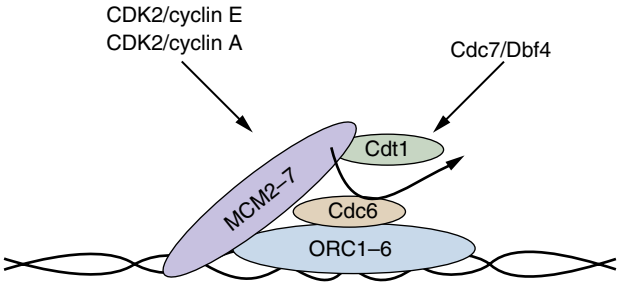


Figure 11-8 Prereplication complex Prereplication complexes (pre-RCs) form during mid- to late G_1 phase, and once they are formed, origins of replication are considered licensed for replication. Origins are recognized first by the hexameric origin of replication complex (ORC1-ORC6), which serves as a landing pad for recruitment of the remaining components. Following ORC recognition, Cdt1 and CDC6 function in a concerted fashion to recruit the MCM2-MCM7 complex, which is considered the replicative helicase. At the beginning of S phase, additional factors (MCM10, CDC45, and polymerases) are recruited and replication can initiate in a fashion dependent on the CDK2 and CDC7 kinase activities.

of *Cdc6* to the ORC. Cdc6 subsequently recruits the MCM complex and Cdt1. However, MCMs are not stably bound at this point. Stable loading of the MCM2-7 helicase complex requires ATP hydrolysis by CDC6, which also results in release of Cdt1.[39] At the G_1/S boundary additional factors are recruited, including *MCM10*, which functions to recruit *Cdc45* and subsequently, DNA polymerase α and primase.

Like G_1 phase, both the G_1/S transition and S-phase progression are driven by cyclin-dependent kinases (CDK2/cyclin E and CDK2/cyclin A, respectively), along with the activity of a distinct CDK-like protein kinase, *Cdc7/Dbf4*. The precise substrates that must be phosphorylated for the firing of origins remain to be conclusively identified. Substrates identified so far include ORC1, MCM2, MCM3, MCM4, and Rad18.[40,41] Not all origins fire simultaneously, but they are temporally regulated. Origins can be grouped generally into those that initiate at the beginning of S phase, "early," and those that fire toward the middle to end of S-phase, "late." The temporal control of firing most likely reflects local controls (chromatin structure modifications) and activation of the complex via phosphorylation.

Paradoxically, although origin firing requires CDK activity, CDK2 activity is also essential for inhibition of a second-round DNA replication (re-replication) within the same cell cycle. Although the precise mechanisms whereby CDK2 prevents replication are still under intense investigation, one way it achieves this goal is through direct regulation of Cdt1 levels. On release from the pre-RC, Cdt1 is subject to ubiquitin-mediated proteolysis. Ubiquitination of Cdt1 is in turn facilitated by CDK2-dependent phosphorylation, which targets it to ubiquitinating machinery.[42] In addition to Cdt1, MCM complexes dissociate from DNA during replication. Whether this dissociation reflects dislodgment from chromatin by polymerases or also reflects a CDK-dependent function remains to be established (Table 11-2).

When DNA is replicated, it is essential to retain memory of epigenetic marks in daughter cells. Histone methylation and acetylation during initiation of DNA replication contribute to this memory as well as proper replication. The histone methyltransferase PR-Set7 has been implicated in catalyzing histone monomethylation at lysine 20 (H4K20 me1) at replication origins, and this methylation is required for initiation of replication.[43] According to a model of epigenetic recruitment at the start of replication, histone acetylation plays a major role in DNA replication origin complex recruitment and H4K12Ac, H3K56Ac accompany the generation of the origin replication complex.[44]

DNA processivity factor proliferating cell nuclear antigen (PCNA) is loaded on both DNA strands during replication and also provides a link between replication and epigenetic memory. NP95 (also referred as UHRF1 and ICBP90) in mammals and VIM1 in *Arabidopsis thaliana* binds to hemimethylated DNA and DNMT1. NP95 recruits DNMT1 to replicating DNA. Another factor, LSH, has been shown to connect DNA methylation to replication in collaboration with NP95. Histone chaperones are required for proper recruitment of histones at DNA. H3.1-H4 chaperone chromatin assembly factor 1 (CAF1) interacts with PCNA, histone deacetylase (HDAC), and Lys methyltransferase. CAF1 regulates nucleosome assembly during DNA replication through proper deposition of H3.1-H4.[45] Another chaperone ASF1 assists CAF1 by supplying newly synthesized histones.[46] Histone methylation levels are transiently reduced during S-phase.[47] It has been recently proposed that TrxG and PcG proteins are associated with DNA during replication.[48]

G_2/M Transition Regulation

The Kinases of Mitosis

The transition from the second Gap phase (G_2) to mitosis (prophase, metaphase, anaphase, telophase) is regulated by CDK1 (formerly Cdc2) in association primarily with cyclin B.[49] Like other CDKs, CDK1 is relatively stable, and activation depends first on accumulation of cyclin B.

Table 11-2 Regulators of DNA Replication and Function

Cdt1	Associates with MCM2-MCM7 and, in concert with Cdc6, facilitates MCM loading on origins.
Cdc6	Functions to recruit and load the MCM complex in an ATPase-dependent manner.
CdC45	Associates with the MCM and is responsible for recruitment of DNA polymerase α, primase, and replication protein A.
MCM2-7	Minichromosome maintenance proteins. Hetero-hexameric complex composed of six distinct but related proteins (MCM2-MCM7). The MCM complex functions as the putative replicative helicase.
MCM10	Structurally distinct from MCM2-MCM7; functions to recruit CDC45.
Orc	Origin recognition complex. Hetero-hexameric complex that binds directly to DNA and functions as a protein landing pad on which the replication complexes form.
Origin	Functionally defined in mammalian cells as regions of chromatin where DNA replication initiates.
Cdc7/Dbf4	The Cdc7 protein kinase, like cyclin-dependent kinases (CDKs), requires an allosteric activator, Dbf4. The Cdc7/Dbf4 kinase phosphorylates components of the replication complexes to initiate DNA replication.
Pre-RC	The prereplication forms during G_1 and contains ORC1-ORC6, Cdc6, MCM2-7. Replication ensues at S phase on recruitment of DNA polymerase and phosphorylation by both the Cdc7/Dbf4 and CDK2-cyclin A protein kinases.

MCM, Minichromosome maintenance; *ORC*, origin of replication complex.

Mitotic cyclins accumulate during S phase and associate with CDK1; however, this complex is maintained in an inactive form via two mechanisms. In the first, Wee/Myt1-dependent phosphorylation of Thr-14/Tyr15 prevents ATP binding. The second mechanism relies on active transport of CDK1/cyclin complexes out of the nucleus. Onset of mitosis is triggered by dephosphorylation of CDK1 by a CDC25 isoform and increased nuclear transport/decreased nuclear exit of CDK1/cyclin complexes. Substrates for CDK1/cyclin B include APC20 (a component of the E3 ligase that ultimately degrades cyclin B), microtubule effectors, microtubule motor proteins, and tubulin itself.[50] From this and related work, it is clear that CDK1-dependent phosphorylation plays a significant role in the formation and regulation of cellular mitotic structures.

In addition to CDK1, a second family of kinases, called polo-like kinases (PLKs), also contributes to mitotic progression. In mammalian cells, there are five PLKs (PLK1-PLK5) with PLK1 being the human homolog of the founding member, Polo, from *Drosophila*.[51] PLKs are serine/threonine kinases. Structurally, they consist of an N-terminal kinase domain and a C terminus with one (PLK3) or two (PLK1-PLK3) "polo box" domains. Current models suggest that PLKs are not constitutively active kinases. Rather, PLK substrates are first phosphorylated by CDKs (e.g., CDK1/cyclin B). Phosphorylation by CDKs is thought to provide a docking site for the polo box domain. Binding of the polo box results in a conformation change in PLK resulting in its activation, whereupon it phosphorylates additional critical residues within the substrate. Alternative models suggest that PLKs are activated through phosphorylation by an upstream kinase, such as CDK1/cyclin B. Although CDK1 can indeed phosphorylate PLK1 in vitro, the functional significance of phosphorylation has not been established. Importantly, neither model is mutually exclusive, and both regulatory mechanisms could contribute to the regulation of PLK activity in cells.

Like CDKs, substrates for PLKs are still being elucidated. As alluded to in previous sections, many PLK substrates may also be CDK substrates. Substrates of PLK1 include CDC25C and Wee1. The consequence of PLK phosphorylation depends on the substrate. Whereas PLK-dependent phosphorylation of CDC25C promotes its activation during mitosis, phosphorylation of Wee1 promotes Wee1 destruction.

Entry into Mitosis

Entry into mitosis requires the nuclear accumulation of active CDK1/cyclin B kinase. During interphase, activity is low. During G_2, cyclin B accumulates as a consequence of increased gene expression and decreased protein degradation. Newly accumulated cyclin B is free to associate with CDK1. However, these complexes are maintained in the cytoplasm and are inactive as a consequence of the combined activities of Wee1 and Myt1. Activation of CDK1/cyclin B at the G_2/M boundary is triggered through CAK-dependent phosphorylation of Thr161 in the T loop and dephosphorylation of Thr14/Tyr15 by CDC25. The initial dephosphorylation is likely catalyzed by CDC25B. The activated CDK1/cyclin B then targets CDC25C and Wee1 to promote CDC25C activity and Wee1 destruction, respectively, thereby forming an amplification loop that drives mitotic progression. The accumulation of CDK1/cyclin B in the nucleus is facilitated by phosphorylation of cyclin B near its nuclear export signal, which thereby impedes nuclear exit. PLK1 contributes to mitotic entry and progression by facilitating these processes. PLK1 can phosphorylate cyclin B just outside the NES (serine 133), thereby preventing nuclear exit. Like the CDK1/cyclin B kinase, PLK1 can also phosphorylate both CDC25C and Wee1, again contributing to CDC25C activation and Wee1 destruction and thereby ensuring full CDK1/cyclin B activation.

Chromosome Cohesion

G_2 phase and the beginning of mitosis are denoted by a 4-N DNA content. Following DNA replication and prior to cell division (cytokinesis), cells must maintain the integrity and proximity of the recently duplicated chromosomes (sister chromatids). Before segregation, sister chromatids are held together or "glued" by a multiprotein complex called Cohesin.[52,53] The cohesin complex ensures that sister chromatids are recognized and properly aligned during metaphase. Once aligned, segregation ensues following proteolytic cleavage of cohesin components. Cohesin is composed of four subunits, Smc1/3 and Scc1/3. Smc1 and Smc3 heterodimerize in a head-to-head, tail-to-tail fashion to form a ring structure in an ATP-dependent manner. The Scc1/3 subunits associate with the Smc heads to complete the structure (Figure 11-9). The Scc1 subunit contacts both Smc1 and 3 and likely stabilizes the ring structure. Models suggest that the cohesin ring has a diameter of approximately 50 nm, sufficiently large to encircle two sister chromatids.[54] Cohesin is envisioned to function by binding and encircling DNA, thereby "gluing" sister chromatids together until released.

Exit from Mitosis

During mitotic prophase, chromosome structures are again altered by a complex called *condensin*, which serves to package chromosomes before mitotic division.[55] The mitotic spindle also forms during prophase. The mitotic spindle is a bilaterally symmetric microtubule organizing center shaped like a football. Each half of the spindle contains a *centrosome* and three distinct sets of microtubules (astral, kinetochore, and polar); the kinetochore microtubules are those

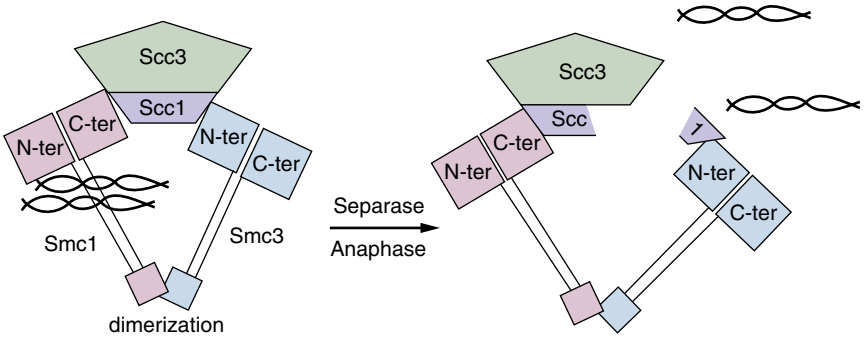


Figure 11-9 Chromosomes are held together by a complex called cohesin Smc1 and Smc3 form a protein ring that is held together by a dimerization "hinge" region that encircles chromatids. The Scc1 and Scc3 subunits interact with the Smc "heads," which retain intrinsic ATPase activity essential for separation of heads to allow DNA to enter. Once all chromatids are aligned during mitosis, Scc1 is cleaved by a protease called Separase to open the ring and allow movement to opposite spindles.

that attach to chromosomes at the kinetochores to facilitate movement to opposite poles before cytokinesis. PLKs are also implicated in the formation of mitotic spindles.[56] Loss-of-function experiments in multiple organisms (yeast to mammalian cells) result in the formation of monopolar spindles. During metaphase, the chromosomes align along the "metaphase plate" in preparation for cell division. Anaphase is marked by segregation of chromosomes to opposite poles. The proteolytic *cleavage* of the Scc1 protein by a protease called *separase* triggers the opening of the cohesin ring, thereby allowing chromosome segregation. Anaphase is also marked by the loss of CDK1 activity, which results from proteolytic destruction of cyclin B and cyclin A. The loss of cohesin and mitotic cyclins is coordinated by a multisubunit E3 ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C; see subsequent sections).

Mitotic Checkpoint

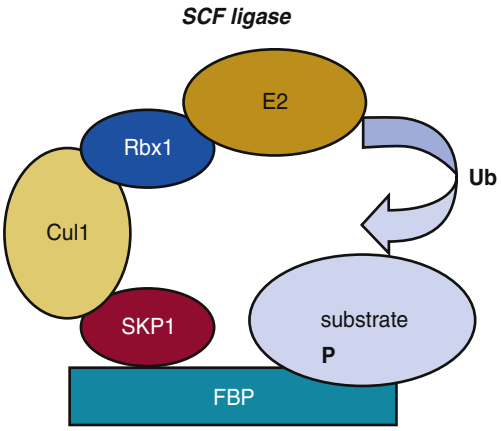
The primary goal of mitosis is to ensure that each daughter cell receives one chromosome complement after cellular division. During mitosis it means that a cell divides only after chromosomes are attached to the microtubules of the mitotic spindle. The *mitotic checkpoint*, or *spindle assembly checkpoint*, is activated as cells enter mitosis, in prometaphase, where it is triggered by *unattached kinetochores*, leading to the delay of anaphase onset. Thus, the role of the proteins that are involved in mitotic checkpoint signaling is to sense the attachment and/or tension at kinetochores.[57] These proteins are often found to be kinetochore-associated and comprise the mitotic checkpoint complex.[58] The mitotic checkpoint complex includes BubR1 and Mps1 kinases, CENP-E (*cen*tromere *p*rotein *E*), Mad (mitotic arrest deficiency proteins)-1 and -2, and others. The mission of mitotic checkpoint kinases is to signal regulatory proteins to inhibit the entry to anaphase. Models suggest that unattached kinetochores lead to phosphorylation of Mad1/2 proteins, which are then directed to the APC/C, resulting in the inhibition of its ubiquitin-ligating activity. This action ensures that chromosomes are accurately distributed to daughter cells. In human neoplasia, the mitotic checkpoint can be inactivated through mutations in components of MCC,[59] contributing to aberrant mitotic divisions and the appearance of aneuploid cells (genetic instability).

Regulated Proteolysis in Cell Cycle Control

Levels of cyclins and CKIs are tightly regulated throughout the cell cycle. This degree of regulation is achieved by coupling the rate of gene expression with regulated proteolysis, which occurs through the ubiquitin proteasome system. The ubiquitin polypeptide consists of 76 residues and is covalently attached to proteins destined for degradation. Attachment occurs through a reversible isopeptide linkage between the carboxy terminus of ubiquitin and lysine residue in the sequence of protein. The name *ubiquitin* derives from early observations of its ubiquitous expression. Indeed, ubiquitin is a highly conserved protein throughout evolution from yeast to humans.

Modification of proteins (*ubiquitination*) with ubiquitin polypeptides requires a conserved series of enzymes. This system includes the ubiquitin-activating enzyme (E1) that performs ATP-dependent activation of ubiquitin. There is only one known E1 enzyme encoded in the human genome. The E1 passes activated ubiquitin to the ubiquitin-conjugating enzyme (E2), of which there are more than 30.[60] In the final stage of ubiquitination, the E2 acts together with an E3, ubiquitin ligase, to attach mono- or polyubiquitin chains onto the target protein. The E3 ligase acts as the specificity factor that determines substrate recognition and thus comprises the largest group. Once a substrate is polyubiquitinated (four or more tandem ubiquitin molecules on a single lysine within the substrate) it is targeted to the 26S proteasome for degradation.

There are two primary E3 ubiquitin ligases involved in the cell cycle that regulate key cell cycle proteins such as cyclins and CKIs. Both sets of ligases belong to a broader E3 subfamily, either the Skp1-Cul1-F-box (SCF) protein ubiquitin ligases or the APC/C. These two systems are structurally similar. However, as one would expect, they target



FBP	Substrate
Skp2	p27, p21, p57
Fbw7	Cyclin E, Myc, Jun
b-Trepl	IkB, b-catenin, Cdc25a
Fbx4	Cyclin D1

Figure 11-10 The Skp1-Cul1-F-box (SCF) E3 ligase F-box protein, or FBP, acts as a specificity component of SCF E3 ligase that recognizes mostly phosphorylated substrates. Further assembly of SKP1-Cul1-Rbx1 components of SCF complex brings E2 ligases and substrates in close proximity for further ubiquitination. Examples of FBPs and their substrates are indicated in the table.

distinct substrates in a cell cycle–specific manner and are differentially regulated.

SCF Ligases

The SCF complex consists of variable and invariable components. The core components employed by all SCF ligases include a scaffold protein Cul1; a ring-finger protein, Rbx1/Roc1; and adaptor protein Skp1 (Figure 11-10). The variable component of the SCF ligase, which determines substrate specificity, is the F-box protein (FBP). FBPs bind Skp1 through an F-box motif initially identified in cyclin F and the substrate bringing the two within close proximity. There are approximately 70 F-box proteins reported in mammals.[61] F-box proteins are classified accordingly to various protein-protein interaction domains that they use to bind to substrates. WD40 repeats give the name to the FBXW class of F-box proteins—leucine-rich repeats (LRRs)—to the FBXL class and F-box proteins that recognize the substrates through other/unknown protein interaction domains belong to the FBXO (F-box only) class. Structurally, FBPs are organized in a fashion that allows them to recognize diverse substrates. Although substrate recognition by FBPs is generally regulated by phosphorylation of the substrate, recognition by one FBP, FBXL2, is determined at least in part by substrate modification with sugar moieties (N-glycans).[62] Thus, the activity of SCF seems to be constant, but the ability to bind to the target protein is regulated.

One of the most rigorously studied FBPs that is involved in cell cycle regulation is Skp2. Although discovered as cyclin A–associated protein, it has since been implicated in the degradation of CKIs: $p27^{Kip1}$, $p21^{Cip1}$, and $p57^{Kip2}$. Skp2 deletion in mice suggests that $p27^{Kip1}$ is a bona fide target for Skp2-mediated degradation, because these mice exhibited striking $p27^{Kip1}$ accumulation.[63] The binding of Skp2 to $p27^{Kip1}$ requires the phosphorylation of Thr187 by cyclin E/A/CDK2 in $p27^{Kip1}$. This binding occurs with high affinity only in the presence of another protein, called Cks1.[64] On binding of $SCF^{skp2/Cks1}$, phosphorylated $p27^{Kip1}$ is ubiquitinated and undergoes proteasome-dependent degradation in late G_1 and early S phases of the cell cycle. Fbw7, another FBP that has been implicated in the degradation of cell cycle key molecules, targets cyclin E, Myc, and c-Jun for degradation.[65] SCF complexes generally regulate proteins involved in G_1 to late S phase, at which point the APC/C is activated and regulates M-phase activities.

FBXO4 is an FBP that specifically directs ubiquitination of cyclin D1. FBXO4 dimerization requires GSK3β-mediated phosphorylation of FBXO4 Ser12, which triggers ligase activation at the G_1/S transition. 14-3-3ε facilitates FBXO4 dimerization, and 14-3-3ε interaction is dependent on Ser8, which is frequently mutated in human cancer, and phosphorylation of Ser12.[66] Recent work revealed the tumor suppressor function of FBXO4. FBXO4 mutations have been identified in human esophageal carcinoma and gastrointestinal stromal tumors.[67,68] Loss of FBXO4 results in cyclin D1 stabilization and nuclear accumulation throughout cell division. $FBXO4^{+/-}$ and $FBXO4^{-/-}$ mice succumb to multiple tumor phenotypes including lymphomas, histiocytic sarcomas, and, less frequently, mammary and hepatocellular carcinomas.[69]

APC/C Ligase

Structurally the APC/C ligase is similar to the SCF complex. The core components are Rbx1/Roc1-related ring-finger protein, APC11, a Cul1-related scaffold protein, APC2, and 11 additional proteins with required but essentially unknown functions.[70] Two components determine substrate specificity similar to SCF FBPs function: cell division cycle 20 (Cdc20) and Cdh1 (Figure 11-11). APC/C ligases recognize specific sequences in target proteins called the destruction box (D-box) and the Ken box. These short-peptide

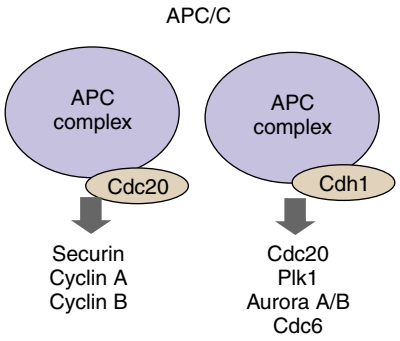


Figure 11-11 The anaphase-promoting complex/cyclosome (APC/C) APC/C ubiquitin ligase is a multiprotein complex that is active in the M through G_1 phases of the cell cycle. The subunits that are responsible for the recognition of substrates by APC are Cdc20 and Cdh1.

sequences are recognized by the Cdh1 and Cdc20 specificity adaptors and therefore facilitate recruitment of the active APC/C.

APC/C is active from anaphase through early G_1 phase. However, the regulation of APC/C activity is distinct from SCF ligases. The Cdc20 subunit of APC/C, APC/C^{Cdc20}, itself undergoes activating phosphorylation events by CDK1/cyclin B. APC/C^{Cdc20} can also be phosphorylated and activated by PLK1 and inactivated by PKA. The activity of APC/C^{Cdc20} is regulated by protein-protein interactions. Mitotic spindle checkpoint proteins Mad1/Mad2 bind to and inhibit APC/C^{Cdc20} function, thereby delaying the onset of anaphase. The substrates of APC/C^{Cdc20} ligase include securin, a protein associated with the mitotic protease separase that allows sister chromatid separation, cyclins A and B. When cyclin B is degraded, CDK1 activity declines, contributing to the activation of APC/C^{Cdh1}; active APC/C^{Cdh1} proceeds to fully ubiquitinate cyclin B molecules, eliminating CDK1 activity. The switch of Cdc20 specificity component of APC/C complex to Cdh1 in late M phase also leads to degradation of Cdc20 itself, Plk1, Aurora A/B kinases, and others (reviewed in).[70] APC/C^{dh1} remains active during early G_1 phase where it also ubiquitinates Skp2, permitting $p27^{Kip1}$ and $p21^{Cip1}$ accumulation, as described earlier.

Sumoylation

Sumoylation is another form of posttranslational modification that regulates the cellular localization of modified proteins. Small ubiquitin-like modifiers (SUMOs) are ubiquitin-like polypeptides that become covalently conjugated to cellular proteins in a manner similar to ubiquitination. Ras induces sumoylation and nuclear accumulation of cyclin D1, thereby inhibiting Rb phosphorylation.[71] The lysine residue, K33, of cyclin D1 is a key site for this newly identified regulation.[71]

Integration of Growth Factor Signals During G_1 Phase by the Ras Small GTP-Binding Protein

Growth factor–dependent signaling promotes the expression and accumulation of factors essential for cell growth (mass accumulation), cell survival, and cell cycle progression. With regard to the cell cycle, growth factor signaling converges on G_1-phase components. Entry to and progression through G_1 phase of the cell cycle requires activation of signal transduction pathways via extracellular growth factors. G_1 progression requires G_1 CDK/cyclin complexes to accumulate and become activated and conversely that CKIs be destroyed. Although this is accomplished through numerous pathways, the molecular basis for Ras-dependent signals in G_1-phase progression is understood with the greatest detail.

Extracellular growth factors promote the guanosine triphosphate (GTP) loading of Ras, its active form. Active Ras-GTP intersects with the cell cycle via the regulation of cyclin D1 expression and activation of the CDK4/6 kinase. Ras-GTP subsequently triggers the activation of multiple independent signaling pathways including canonical MAP kinase signaling Raf, mitogen-activated protein kinase-kinases (MEK1 and -2), and the sustained activation of extracellular signal-regulated protein kinases (ERKs or MAPK). This pathway contributes to cyclin D1 gene expression.[72] Ras-GTP triggers the activation of a second related, small-GTP binding protein, Rho; activation of Rho also plays a critical role in growth factor–dependent cyclin D1 expression during G_1 phase. A third pathway activated by Ras involves PI-3K and Akt (PKB). The activation of this pathway contributes to increased translation of a multitude of proteins, including cyclin D1 by virtue of the ability of Akt to regulate translation initiation.[73] Active Akt also inactivates glycogen synthase kinase-3β (GSK-3β) by site-specific phosphorylation. Active GSK-3β kinase phosphorylates cyclin D1, thereby promoting cyclin D1 ubiquitination and proteolysis.[74] Thus inactivation of GSK-3β is a critical step necessary for cyclin D1 accumulation during G_1 phase.

For cells to progress through G_1 phase, growth factor signaling must promote increased G_1 cyclin accumulation and suppress accumulation of the cell cycle inhibitor $p27^{Kip1}$. Active Ras also plays a central role in the regulation of $p27^{Kip1}$ in G_1 phase by decreasing the efficiency of $p27^{Kip1}$ translation and increasing the kinetics of $p27^{Kip1}$ proteolysis. Ras-dependent regulation of $p27^{Kip1}$ translation and degradation requires Rho signaling. The concerted increase in cyclin D1 accumulation and decrease in $p27^{Kip1}$ accumulation provides a threshold of CDK4/cyclin D1 activity that is necessary and sufficient for restriction point passage and commitment to S-phase entry.

Deregulation of G_1 Restriction Point Control in Cancer

In G_1 phase, cells make the decision to either progress through the restriction point and enter S phase or enter G_0. These decisions are based on extracellular signals that the cell receives and on the integrity of signaling machinery that detects these signals. Deregulation of G_1 progression is a frequent occurrence in cancer, through mutations or deregulated expression of CDKs, cyclins, or CKIs. Loss- or gain-of-function mutations in upstream regulators of the CDK kinases also occur in cancer. In this section, we discuss some alterations found in cell cycle regulators in cancer.

Cyclin D–dependent kinases are a primary point of control for the progression through G_1 phase and are linked to cancer progression. Cyclin D1 overexpression is a hallmark of breast and esophageal cancers.[75] In many cases this upregulation is due to cyclin D1 gene amplifications, but it can also result from increased transcription.[75] In addition to gene expression alterations, decreased cyclin D1 proteolysis is implicated in deregulated cyclin D/CDK4 activity in breast and esophageal cancers. Cyclin D1 overexpression also occurs as a consequence of chromosomal translocations. Amplifications encompassing the *CDK4* and *CDK2* genes have been reported in large B-cell lymphomas, lung tumors, and cervical carcinomas. Downstream targets of cyclin D/CDK4/6 kinases, Rb proteins, are also targeted in cancer. Mutations and deletions in the *Rb* gene are common events in tumors; inactivation of Rb alleviates a cell need for CDK4/6 kinase and thus relieves some cellular dependence on growth factor signals.[76]

As one might anticipate, Cip/Kip inhibitors can also function as tumor suppressor proteins in mouse model systems, and, consistent with this work, their expression is deregulated in human cancers. p53, the main transcriptional regulator of $p21^{Cip1}$, is often lost or mutated during tumorigenesis. Reduced $p27^{Kip1}$ levels alone or together with increased cyclin E expression are associated with poor prognosis in breast and ovarian carcinomas. Inactivation of $p16^{Ink4a}$ occurs frequently in lung, bladder, and breast carcinomas, as well as leukemia (reviewed in Ref. 24).

In addition to alterations in the expression and integrity of cell cycle genes in cancers, attenuation of their regulatory pathways also occurs. These include signaling pathways (Ras), transcription factors (myc), and components of ubiquitin ligases. Skp2, the specificity component of the SCF ligase for $p27^{Kip1}$, is upregulated in a variety of tumors, including colon, lung, breast, prostate, and lymphoma,[70] where it decreases $p27^{Kip1}$. Another F-box protein, Fbw7, which regulates degradation of cyclin E, is mutated in ovarian and breast cancers.

Altered functionality of cyclin D1 ubiquitin ligase can lead to increased cyclin D1 expression and ultimately to tumorigenesis. Cyclin ubiquitination requires both FBXO4 and a specificity co-factor, αB-crystallin.[77] αB-crystallin expression is lost or downregulated in breast cancer and melanoma cell lines, which correlates with decreased cyclin D1 proteolysis.[78,79] Primary esophageal carcinomas, which are known to frequently overexpress cyclin D1, exhibit hemizygous, missense mutations of FBXO4.[80]

Mutations and deregulation of the expression of regulators of mitosis are also observed in human malignancy. Increased accumulation of Cdc20 (APC/C) is observed in lung and gastric tumor cell lines. Mutations in *PLK1* are found in human cancer cell lines, and its attenuated expression is observed in colorectal, endometrial, and breast carcinomas.

Targeting the Cell Cycle as a Therapeutic Modality

Dysregulated cell division is a hallmark of cancer progression.[81] Therefore, the use of agents targeting the cell cycle machinery has long been considered as an ideal strategy for cancer therapy.[82] Cell cycle–based agents can be grouped into categories that reflect their molecular targets: CDK inhibitors, checkpoint inhibitors, and mitotic inhibitors. These drugs target the abnormal expression of CDKs, mitotic kinases/kinesins, or affect the cellular checkpoints, resulting in cell-cycle arrest followed by induction of apoptosis in cancer cells.

Targeting CDKs

The rationale for targeting CDKs in anticancer therapy is based on both their role in catalyzing cell division and, in certain cases, the frequency of their perturbation in human malignancy.[83] The rationale suggests that inhibition of CDKs would selectively block tumor growth without compromising normal cells, given that most normal tissues are postmitotic. During the past two decades, numerous CDK inhibitors have been identified as antitumor agents. These drugs have been classified as pan-CDK inhibitors or selective CDK inhibitors. First-generation CDK inhibitors such as flavopiridol, olomoucine, and roscovitine generally did not meet expectations following preclinical studies, showing low activity or no response in the clinical trials. Second-generation CDK inhibitors, such as aminothiazole SNS-032, pyrazole-3-carboxamide AT7519, and synthetic flavone P276-00, have recently been shown to be promising drug candidates in preclinical and clinical trials.

SNS-032, a potent and selective CDK 2, 7, and 9 inhibitor, is currently in phase I clinical study in B-cell malignancies including chronic lymphocytic leukemia, mantle-cell lymphoma (MCL), and multiple myeloma, providing data supporting the ongoing clinical trials. A phase I study of

pan-CDK inhibitor AT7519 was carried out in 28 patients with refractory solid tumors. Four patients showed stable disease and one had a prolonged partial response.[84] A phase II study to treat patients with relapsed or refractory multiple myeloma has been initiated. P276-00 is a highly specific inhibitor of CDK2. Confirmed stable disease has been observed in a phase I study in patients with advanced refractory neoplasms. Phase I/II studies are being performed to evaluate P276-00 efficacy in combination with gemcitabine in patients with pancreatic cancers or in combination with radiation in patients with squamous-cell carcinoma of the head and neck.

Other selective inhibitors such as CDK4/6 inhibitor fascaplysin, CDK4 inhibitor ryuvidine, CDK2 inhibitors purvalanol A and NU2058, and CDK5 inhibitor BML-259 are commercially available and broadly used in the research; however, they had not been entered into clinical trials. The highly selective CDK4/6 inhibitor PD 0332991 is a novel, orally administered inhibitor, which shows potential single-agent activity in clinical trials. PD 0332991 induces G_1 cell cycle arrest by blocking phosphorylation of Rb at CDK4/6-specific sites. Phase I study has been conducted in patients with Rb-expressing advanced solid tumors, relapsed MCL, or refractory non-Hodgkin's lymphoma. Dose-limiting toxicities relate mainly to myelosuppression.[85-87] A therapeutic response to PD 0332991 has also been seen in inoperable growing teratoma.[88] A phase II study of PD 0332991 in patients with recurrent Rb-positive glioblastoma is currently ongoing.

Targeting Cell Cycle Checkpoints

Targeting the S and G_2 checkpoints is also attractive for cancer therapy because loss of G_1 checkpoint control is a common feature of cancer cells,[89] making them more reliant on the S and G_2 checkpoints to prevent DNA damage–triggered cell death. Various molecules such as CHK1, CHK2, PP2A, Wee1, and cell division cycle 25 (CDC25) have been suggested as the key targets for checkpoint abrogation.[90] Numerous checkpoint inhibitors have entered into clinical trials, most of which focused on CHK1. Among all the checkpoint inhibitors, UCN-01 is most clinically advanced,[91-93] but after phase II trials it was discontinued because of dose-limiting toxicities and a lack of convincing efficacy. The newer, more specific inhibitors of CHK are still under investigation.[90,94]

Targeting Regulators of Mitosis

Critical mediators of mitosis that are also implicated in tumorigenesis include three distinct protein kinase families (CDKs, Aurora kinases, and PLKs) and specific mitotic kinesins. Overexpression/amplification of Aurora kinases has been observed in cancer cells and contributes to dysregulated spindle formation, compromised spindle checkpoint, and cytokinesis failure; collectively, these anomalies contribute to cellular aneuploidy.[95] Aurora kinase inhibitors are being pursued, and many of them are already in clinical development.

Like Aurora kinases, PLK1 is often overexpressed in human tumors but not in healthy, nondividing cells. This makes PLK inhibitors an attractive, selective target for cancer drug development. PLK inhibitors interfere with different stages of mitosis, such as centrosome maturation, spindle formation, chromosome separation, and cytokinesis. They induce mitotic chaos and severely perturb cell cycle progression, eventually leading to cancer cell death.[96] Numerous PLK inhibitors are being tested to evaluate their therapeutic potential in oncology.

Kinesins either have a single function or are functionally involved at different stages of the mitotic phase, making them potential anticancer targets.[97] Compounds that inhibit mitotic kinesins EG5 and centromere-associated protein E (CENP-E) have entered clinical trials. Additional mitotic kinesins are currently at varying stages of drug development, raising the possibility of kinesin as a successful target for cancer therapy.

Conclusions

Significant advances have been made in the understanding of the molecular basis of cell cycle regulation. Conceptually, it was anticipated that understanding the basic mechanisms and regulators would permit scientists to ask how they contribute to organismal development and/or cancer progression. Indeed, these questions are now being addressed through targeted deletion of individual genes in the mouse genome. G_1 cyclins and CDKs have been removed from the mouse genome by targeted deletion to evaluate the role of these molecules in organismal development and basic cell growth. Although genetic deletion strains in mice have revealed unique properties of each molecule, what has been most striking is the revelation that no one cyclin or CDK is absolutely essential for development.[98] Thus, although each mammalian CDK is considered to have distinct substrates, in an intact cell there is sufficient redundancy to permit loss of any one complex.

The identification of the critical regulators of cell division has also facilitated the development of antiproliferative therapies through the design of small-molecule inhibitors of the CDKs. Given that deregulated growth control is a fundamental property of cancer, the development of small molecules that inhibit the molecular machine that drives cell cycle transitions is a conceptually attractive therapeutic option. The continued investigation of components of the cell cycle machine will undoubtedly continue to contribute fundamental insights into cell growth control and potentially provide additional insights into diseases that alter growth properties.

Key References

14. Ferguson AM, White LS, Donovan PJ, et al. Normal cell cycle and checkpoint responses in mice and cells lacking Cdc25B and Cdc25C protein phosphatases. *Mol Cell Biol*. 2005;25:2853-2860.
28. Roeb W, Boyer A, Cavenee WK, et al. PAX3-FOXO1 controls expression of the p57Kip2 cell-cycle regulator through degradation of EGR1. *Proc Natl Acad Sci U S A*. 2007;104:18085-18090.
29. Merlo P, Fulco M, Costanzo A, et al. A role of p73 in mitotic exit. *J Biol Chem*. 2005;280:30354-30360.
36. Vlieghe K, Boudolf V, Beemster GT, et al. The DP-E2F-like gene DEL1 controls the endocycle in *Arabidopsis thaliana*. *Curr Biol*. 2005;15:59-63.
37. Li J, Ran C, Li E, et al. Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. *Dev Cell*. 2008;14:62-75.
40. Lin DI, Aggarwal P, Diehl JA. Phosphorylation of MCM3 on Ser-112 regulates its incorporation into the MCM2-7 complex. *Proc Natl Acad Sci U S A*. 2008;105:8079-8084.
41. Vaziri C, Masai H. Integrating DNA replication with trans-lesion synthesis via Cdc7. *Cell Cycle*. 2010;9:4818-4823.
42. Li X, Zhao Q, Liao R, et al. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J Biol Chem*. 2003;278:30854-30858.
43. Tardat M, Brustel J, Kirsh O, et al. The histone H4 Lys 20 methyltransferase PR-Set7 regulates replication origins in mammalian cells. *Nat Cell Biol*. 2010;12:1086-1093.
44. Liu J, McConnell K, Dixon M, et al. Analysis of model replication origins in *Drosophila* reveals new aspects of the chromatin landscape and its relationship to origin activity and the prereplicative complex. *Mol Biol Cell*. 2011;23:200-212.
45. Polo SE, Roche D, Almouzni G. New histone incorporation marks sites of UV repair in human cells. *Cell*. 2006;127:481-493.
47. Xu M, Wang W, Chen S, et al. A model for mitotic inheritance of histone lysine methylation. *EMBO Rep*. 2012;13:60-67.
48. Petruk S, Sedkov Y, Johnston DM, et al. TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. *Cell*. 2012;150:922-933.
54. Ivanov D, Nasmyth K. A topological interaction between cohesin rings and a circular minichromosome. *Cell*. 2005;122:849-860.
58. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*. 1999;13:1501-1512.
60. Zhang XD, Matunis MJ. Ub in charge: regulating E2 enzyme nuclear import. *Nat Cell Biol*. 2005;7:12-14.
61. Nakayama KI, Nakayama K. Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin Cell Dev Biol*. 2005;16:323-333.
65. Tetzlaff MT, Yu W, Li M, et al. Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein. *Proc Natl Acad Sci U S A*. 2004;101:3338-3345.
66. Barbash O, Lee EK, Diehl JA. Phosphorylation-dependent regulation of SCF(Fbx4) dimerization and activity involves a novel component, 14-3-3varepsilon. *Oncogene*. 2011;30:1995-2002.
67. Barbash O, Zamfirova P, Lin DI, et al. Mutations in Fbx4 inhibit dimerization of the SCF(Fbx4) ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell*. 2008;14:68-78.
68. Kang G, Lee J, Jang KT, et al. Multiplex mutation screening by mass spectrometry in gastrointestinal stromal tumours. *Pathology*. 2012;44:460-464.
69. Vaites LP, Lee EK, Lian Z, et al. The Fbx4 tumor suppressor regulates cyclin D1 accumulation and prevents neoplastic transformation. *Mol Cell Biol*. 2011;31:4513-4523.
70. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer*. 2006;6:369-381.
71. Wang XD, Lapi E, Sullivan A, et al. SUMO-modified nuclear cyclin D1 bypasses Ras-induced senescence. *Cell Death Differ*. 2006;18:304-314.
73. Shi Y, Sharma A, Wu H, et al. Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. *J Biol Chem*. 2005;280:10964-10973.
77. Lin DI, Barbash O, Kumar KG, et al. Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex. *Mol Cell*. 2006;24:355-366.
78. Hu R, Aplin AE. AlphaB-crystallin is mutant B-RAF regulated and contributes to cyclin D1 turnover in melanocytic cells. *Pigment Cell Melanoma Res*. 2011;23:201-209.
79. Chelouche-Lev D, Kluger HM, Berger AJ, et al. AlphaB-crystallin as a marker of lymph node involvement in breast carcinoma. *Cancer*. 2004;100:2543-2548.
80. Barbash O, Diehl JA. SCF(Fbx4/alphaB-crystallin) E3 ligase: when one is not enough. *Cell Cycle*. 2008;7:2983-2986.
81. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
82. Schwartz GK, Shah MA. Targeting the cell cycle: a new approach to cancer therapy. *J Clin Oncol*. 2005;23:9408-9421.
84. Mahadevan D, Plummer R, Squires MS, et al. A phase I pharmacokinetic and pharmacodynamic study of AT7519, a cyclin-dependent kinase inhibitor in patients with refractory solid tumors. *Ann Oncol*. 2011;22:2137-2143.
85. Leonard JP, LaCasce AS, Smith MR, et al. Selective CDK4/6 inhibition with tumor responses by PD0332991 in patients with mantle cell lymphoma. *Blood*. 2012;119:4597-4607.
86. Flaherty KT, Lorusso PM, Demichele A, et al. Phase I, dose-escalation trial of the oral cyclin-dependent kinase 4/6 inhibitor PD 0332991, administered using a 21-day schedule in patients with advanced cancer. *Clin Cancer Res*. 2012;18:568-576.
87. Schwartz GK, LoRusso PM, Dickson MA, et al. Phase I study of PD 0332991, a cyclin-dependent kinase inhibitor, administered in 3-week cycles (Schedule 2/1). *Br J Cancer*. 2011;104:1862-1868.
88. Vaughn DJ, Flaherty K, Lal P, et al. Treatment of growing teratoma syndrome. *N Engl J Med*. 2009;360:423-424.
89. Massague J. G1 cell-cycle control and cancer. *Nature*. 2004;432:298-306.
90. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. *Nat Rev Cancer*. 2012;12:801-817.
91. Li T, Christensen SD, Frankel PH, et al. A phase II study of cell cycle inhibitor UCN-01 in patients with metastatic melanoma: a California Cancer Consortium trial. *Invest New Drugs*. 2012;30:741-748.
92. Fracasso PM, Williams KJ, Chen RC, et al. A Phase 1 study of UCN-01 in combination with irinotecan in patients with resistant solid tumor malignancies. *Cancer Chemother Pharmacol*. 2011;67:1225-1237.
93. Kummar S, Gutierrez ME, Gardner ER, et al. A phase I trial of UCN-01 and prednisone in patients with refractory solid tumors and lymphomas. *Cancer Chemother Pharmacol*. 2010;65:383-389.
94. Garrett MD, Collins I. Anticancer therapy with checkpoint inhibitors: what, where and when? *Trends Pharmacol Sci*. 2011;32:308-316.
95. Cheung CH, Coumar MS, Chang JY, et al. Aurora kinase inhibitor patents and agents in clinical testing: an update (2009-10). *Expert Opin Ther Pat*. 2011;21:857-884.
96. Schoffski P. Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. *Oncologist*. 2009;14:559-570.
97. Rath O, Kozielski F. Kinesins and cancer. *Nat Rev Cancer*. 2012;12:527-539.
98. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*. 2004;18:2699-2711.

References

1. Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol*. 1991;115:1-17.
2. Brown NR, Noble ME, Endicott JA, et al. The crystal structure of cyclin A. *Structure*. 1995;3:1235-1247.
3. Jeffrey PD, Russo AA, Polyak K, et al. Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature*. 1995;376:313-320.
4. Makela TP, Tassan JP, Nigg EA, et al. A cyclin associated with the CDK-activating kinase MO15. *Nature*. 1994;371:254-257.
5. Shiekhattar R, Mermelstein F, Fisher RP, et al. Cdk-activating kinase complex is a component of human transcription factor TFIIH. *Nature*. 1995;374:283-287.
6. Feaver WJ, Svejstrup JQ, Henry NL, et al. Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. *Cell*. 1994;79:1103-1109.
7. Kaldis P, Sutton A, Solomon MJ. The Cdk-activating kinase (CAK) from budding yeast. *Cell*. 1996;86:553-564.
8. Espinoza FH, Farrell A, Erdjument-Bromage H, et al. A cyclin-dependent kinase-activating kinase (CAK) in budding yeast unrelated to vertebrate CAK. *Science*. 1996;273:1714-1717.
9. Baldin V, Ducommun B. Subcellular localisation of human wee1 kinase is regulated during the cell cycle. *J Cell Sci. 108 Part*. 1995;6:2425-2432.
10. Atherton-Fessler S, Parker LL, Geahlen RL, et al. Mechanisms of p34cdc2 regulation. *Mol Cell Biol*. 1993;13:1675-1685.
11. Terada Y, Tatsuka M, Jinno S, et al. Requirement for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation. *Nature*. 1995;376:358-362.
12. Nilsson I, Hoffmann I. Cell cycle regulation by the Cdc25 phosphatase family. *Prog Cell. Cycle Res*. 2000;4:107-114.
13. Sandhu C, Donovan J, Bhattacharya N, et al. Reduction of Cdc25A contributes to cyclin E1-Cdk2 inhibition at senescence in human mammary epithelial cells. *Oncogene*. 2000;19:5314-5323.
14. Ferguson AM, White LS, Donovan PJ, et al. Normal cell cycle and checkpoint responses in mice and cells lacking Cdc25B and Cdc25C protein phosphatases. *Mol Cell Biol*. 2005;25:2853-2860.
15. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*. 1999;13:1501-1512.
16. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. 1993;366:704-707.
17. Brotherton DH, Dhanaraj V, Wick S, et al. Crystal structure of the complex of the cyclin D-dependent kinase Cdk6 bound to the cell-cycle inhibitor p19INK4d. *Nature*. 1998;395:244-250.
18. Boice JA, Fairman R. Structural characterization of the tumor suppressor p16, an ankyrin-like repeat protein. *Protein Sci*. 1996;5:1776-1784.
19. Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997;88:593-602.
20. Shapiro GI, Edwards CD, Kobzik L, et al. Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines. *Cancer Res*. 1995;55:505-509.
21. Thullberg M, Bartkova J, Khan S, et al. Distinct versus redundant properties among members of the INK4 family of cyclin-dependent kinase inhibitors. *FEBS Lett*. 2000;470:161-166.
22. Quelle DE, Zindy F, Ashmun RA, et al. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell*. 1995;83:993-1000.
23. Sherr CJ. Tumor surveillance via the ARF-p53 pathway. *Genes Dev*. 1998;12:2984-2991.
24. Serrano M, Lee H, Chin L, et al. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*. 1996;85:27-37.
25. Chen J, Jackson PK, Kirschner MW, et al. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature*. 1995;374:386-388.
26. Russo AA, Jeffrey PD, Patten AK, et al. Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature*. 1996;382:325-331.
27. Polyak K, Kato JY, Solomon MJ, et al. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev*. 1994;8:9-22.
28. Roeb W, Boyer A, Cavenee WK, et al. PAX3-FOXO1 controls expression of the p57Kip2 cell-cycle regulator through degradation of EGR1. *Proc Natl Acad Sci U S A*. 2007;104:18085-18090.
29. Merlo P, Fulco M, Costanzo A, et al. A role of p73 in mitotic exit. *J Biol Chem*. 2005;280:30354-30360.
30. Kovesdi I, Reichel R, Nevins JR. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proc Natl Acad Sci U S A*. 1987;84:2180-2184.
31. Yee AS, Reichel R, Kovesdi I, et al. Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. *EMBO J*. 1987;6:2061-2068.
32. Johnson DG, Schwarz JK, Cress WD, et al. Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature*. 1993;365:349-352.
33. Ohtani K, DeGregori J, Nevins JR. Regulation of the cyclin E gene by transcription factor E2F1. *Proc Natl Acad Sci U S A*. 1995;92:12146-12150.
34. Pierce AM, Schneider-Broussard R, Philhower JL, et al. Differential activities of E2F family members: unique functions in regulating transcription. *Mol Carcinog*. 1998;22:190-198.
35. Kato J, Matsushime H, Hiebert SW, et al. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev*. 1993;7:331-342.
36. Vlieghe K, Boudolf V, Beemster GT, et al. The DP-E2F-like gene DEL1 controls the endocycle in *Arabidopsis thaliana*. *Curr Biol*. 2005;15:59-63.
37. Li J, Ran C, Li E, et al. Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. *Dev Cell*. 2008;14:62-75.
38. Sherr CJ, Matsushime H, Roussel MF. Regulation of CYL/cyclin D genes by colony-stimulating factor 1. *Ciba Found Symp*. 1992;170:209-219; discussion 219-226.
39. Tye BK. MCM proteins in DNA replication. *Annu Rev Biochem*. 1999;68:649-686.
40. Lin DI, Aggarwal P, Diehl JA. Phosphorylation of MCM3 on Ser-112 regulates its incorporation into the MCM2-7 complex. *Proc Natl Acad Sci U S A*. 2008;105:8079-8084.
41. Vaziri C, Masai H. Integrating DNA replication with trans-lesion synthesis via Cdc7. *Cell Cycle*. 2010;9:4818-4823.
42. Li X, Zhao Q, Liao R, et al. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J Biol Chem*. 2003;278:30854-30858.
43. Tardat M, Brustel J, Kirsh O, et al. The histone H4 Lys 20 methyltransferase PR-Set7 regulates replication origins in mammalian cells. *Nat Cell Biol*. 2010;12:1086-1093.
44. Liu J, McConnell K, Dixon M, et al. Analysis of model replication origins in *Drosophila* reveals new aspects of the chromatin landscape and its relationship to origin activity and the prereplicative complex. *Mol Biol Cell*. 2011;23:200-212.
45. Polo SE, Roche D, Almouzni G. New histone incorporation marks sites of UV repair in human cells. *Cell*. 2006;127:481-493.
46. Mello JA, Sillje HH, Roche DM, et al. Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. *EMBO Rep*. 2002;3:329-334.
47. Xu M, Wang W, Chen S, et al. A model for mitotic inheritance of histone lysine methylation. *EMBO Rep*. 2012;13:60-67.
48. Petruk S, Sedkov Y, Johnston DM, et al. TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. *Cell*. 2012;150:922-933.
49. Morgan DO. Principles of CDK regulation. *Nature*. 1995;374:131-134.
50. Kotani S, Tanaka H, Yasuda H, et al. Regulation of APC activity by phosphorylation and regulatory factors. *J Cell Biol*. 1999;146:791-800.
51. Clay FJ, McEwen SJ, Bertoncello I, et al. Identification and cloning of a protein kinase-encoding mouse gene, Plk, related to the polo gene of. *Drosophila. Proc Natl Acad Sci U S A*. 1993;90:4882-4886.
52. Uhlmann F, Lottspeich F, Nasmyth K. Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*. 1999;400:37-42.
53. Nasmyth K. Separating sister chromatids. *Trends Biochem Sci*. 1999;24:98-104.
54. Ivanov D, Nasmyth K. A topological interaction between cohesin rings and a circular minichromosome. *Cell*. 2005;122:849-860.

55. Hirano T, Kobayashi R, Hirano M. Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a *Xenopus* homolog of the *Drosophila* Barren protein. *Cell*. 1997;89:511-521.
56. Lee KS, Yuan YL, Kuriyama R, et al. Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. *Mol Cell Biol*. 1995;15:7143-7151.
57. Ault JG, Nicklas RB. Tension, microtubule rearrangements, and the proper distribution of chromosomes in mitosis. *Chromosoma*. 1989;98:33-39.
58. Foley EA, Kapoor TM. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nat Rev Mol Cell Biol*. 2013;14:25-37.
59. Cahill DP, Lengauer C, Yu J, et al. Mutations of mitotic checkpoint genes in human cancers. *Nature*. 1998;392:300-303.
60. Zhang XD, Matunis MJ. Ub in charge: regulating E2 enzyme nuclear import. *Nat Cell Biol*. 2005;7:12-14.
61. Nakayama KI, Nakayama K. Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin Cell Dev Biol*. 2005;16:323-333.
62. Yoshida Y, Chiba T, Tokunaga F, et al. E3 ubiquitin ligase that recognizes sugar chains. *Nature*. 2002;418:438-442.
63. Nakayama K, Nagahama H, Minamishima YA, et al. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J*. 2000;19:2069-2081.
64. Spruck C, Strohmaier H, Watson M, et al. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. *Mol Cell*. 2001;7:639-650.
65. Tetzlaff MT, Yu W, Li M, et al. Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein. *Proc Natl Acad Sci U S A*. 2004;101:3338-3345.
66. Barbash O, Lee EK, Diehl JA. Phosphorylation-dependent regulation of SCF(Fbx4) dimerization and activity involves a novel component, 14-3-3varepsilon. *Oncogene*. 2011;30:1995-2002.
67. Barbash O, Zamfirova P, Lin DI, et al. Mutations in Fbx4 inhibit dimerization of the SCF(Fbx4) ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell*. 2008;14:68-78.
68. Kang G, Lee J, Jang KT, et al. Multiplex mutation screening by mass spectrometry in gastrointestinal stromal tumours. *Pathology*. 2012;44:460-464.
69. Vaites LP, Lee EK, Lian Z, et al. The Fbx4 tumor suppressor regulates cyclin D1 accumulation and prevents neoplastic transformation. *Mol Cell Biol*. 2011;31:4513-4523.
70. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer*. 2006;6:369-381.
71. Wang XD, Lapi E, Sullivan A, et al. SUMO-modified nuclear cyclin D1 bypasses Ras-induced senescence. *Cell Death Differ*. 2006;18:304-314.
72. Filmus J, Robles AI, Shi W, et al. Induction of cyclin D1 overexpression by activated ras. *Oncogene*. 1994;9:3627-3633.
73. Shi Y, Sharma A, Wu H, et al. Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. *J Biol Chem*. 2005;280:10964-10973.
74. Diehl JA, Cheng M, Roussel MF, et al. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*. 1998;12:3499-3511.
75. Steeg PS, Zhou Q. Cyclins and breast cancer. *Breast Cancer Res Treat*. 1998;52:17-28.
76. Hall M, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv Cancer Res*. 1996;68:67-108.
77. Lin DI, Barbash O, Kumar KG, et al. Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF(FBX4-alphaB crystallin) complex. *Mol Cell*. 2006;24:355-366.
78. Hu R, Aplin AE. AlphaB-crystallin is mutant B-RAF regulated and contributes to cyclin D1 turnover in melanocytic cells. *Pigment Cell Melanoma Res*. 2011;23:201-209.
79. Chelouche-Lev D, Kluger HM, Berger AJ, et al. AlphaB-crystallin as a marker of lymph node involvement in breast carcinoma. *Cancer*. 2004;100:2543-2548.
80. Barbash O, Diehl JA. SCF(Fbx4/alphaB-crystallin) E3 ligase: when one is not enough. *Cell Cycle*. 2008;7:2983-2986.
81. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
82. Schwartz GK, Shah MA. Targeting the cell cycle: a new approach to cancer therapy. *J Clin Oncol*. 2005;23:9408-9421.
83. Chen YN, Sharma SK, Ramsey TM, et al. Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. *Proc Natl Acad Sci U S A*. 1999;96:4325-4329.
84. Mahadevan D, Plummer R, Squires MS, et al. A phase I pharmacokinetic and pharmacodynamic study of AT7519, a cyclin-dependent kinase inhibitor in patients with refractory solid tumors. *Ann Oncol*. 2011;22:2137-2143.
85. Leonard JP, LaCasce AS, Smith MR, et al. Selective CDK4/6 inhibition with tumor responses by PD0332991 in patients with mantle cell lymphoma. *Blood*. 2012;119:4597-4607.
86. Flaherty KT, Lorusso PM, Demichele A, et al. Phase I, dose-escalation trial of the oral cyclin-dependent kinase 4/6 inhibitor PD 0332991, administered using a 21-day schedule in patients with advanced cancer. *Clin Cancer Res*. 2012;18:568-576.
87. Schwartz GK, LoRusso PM, Dickson MA, et al. Phase I study of PD 0332991, a cyclin-dependent kinase inhibitor, administered in 3-week cycles (Schedule 2/1). *Br J Cancer*. 2011;104:1862-1868.
88. Vaughn DJ, Flaherty K, Lal P, et al. Treatment of growing teratoma syndrome. *N Engl J Med*. 2009;360:423-424.
89. Massague J. G1 cell-cycle control and cancer. *Nature*. 2004;432:298-306.
90. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. *Nat Rev Cancer*. 2012;12:801-817.
91. Li T, Christensen SD, Frankel PH, et al. A phase II study of cell cycle inhibitor UCN-01 in patients with metastatic melanoma: a California Cancer Consortium trial. *Invest New Drugs*. 2012;30:741-748.
92. Fracasso PM, Williams KJ, Chen RC, et al. A Phase 1 study of UCN-01 in combination with irinotecan in patients with resistant solid tumor malignancies. *Cancer Chemother Pharmacol*. 2011;67:1225-1237.
93. Kummar S, Gutierrez ME, Gardner ER, et al. A phase I trial of UCN-01 and prednisone in patients with refractory solid tumors and lymphomas. *Cancer Chemother Pharmacol*. 2010;65:383-389.
94. Garrett MD, Collins I. Anticancer therapy with checkpoint inhibitors: what, where and when? *Trends Pharmacol Sci*. 2011;32:308-316.
95. Cheung CH, Coumar MS, Chang JY, et al. Aurora kinase inhibitor patents and agents in clinical testing: an update (2009-10). *Expert Opin Ther Pat*. 2011;21:857-884.
96. Schoffski P. Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. *Oncologist*. 2009;14:559-570.
97. Rath O, Kozielski F. Kinesins and cancer. *Nat Rev Cancer*. 2012;12:527-539.
98. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev*. 2004;18:2699-2711.

David A. Guertin and David M. Sabatini

12

Cell Growth

What Is Cell Growth?

Cell growth is the process by which cells accumulate mass and increase in physical size. On average, dividing animal cells are approximately 10 to 20 μm in diameter. Terminally differentiated cells have a wide range of sizes, spanning from tiny red blood cells (~5 μm in diameter) to motor neurons, which can grow to hundreds of micrometers in length.[1] For a typical dividing cell, water accounts for about 70% of the weight of a cell, and macromolecules, such as nucleic acids, proteins, polysaccharides, and lipids constitute most of the remaining mass (~25%—trace amounts of ions and small molecules make up the difference). The largest contribution to cellular dry mass is typically from proteins, which makes up about 18% of the total cell weight on average. There are many physical, chemical, and biological factors that affect the biosynthesis of macromolecules and therefore final cell size. Intracellular signaling networks that regulate metabolism and control macromolecule biosynthesis are particularly relevant to cancer. As discussed later, deregulation of the cellular circuitry controlling biomass accumulation is associated with a wide spectrum of human cancers.

There are many different examples in nature of how cells can grow. In some cases, cell size is proportional to DNA content. For instance, continued DNA replication in the absence of cell division (called *endoreplication*) results in increased cell size. Megakaryoblasts, which mature into granular megakaryocytes, the platelet-producing cells of bone marrow, typically grow this way. These cells cease division and then undergo multiple rounds of DNA synthesis, increasing from about 20 μm to approximately 100 μm in diameter as a result of the increased DNA content. It is unclear whether increased DNA content simply leads to an increase in total cellular material or whether cells actively grow to cope with the larger genome size. This growth strategy is found throughout nature in animals, plants, and single-celled organisms. By a different strategy, adipocytes can grow to approximately 85 to 120 μm by accumulating intracellular lipids. In contrast to endoreplication or lipid accumulation, some terminally differentiated cells, such as neurons and cardiac muscle cells, cease dividing and grow without increasing their DNA content. These cells proportionately increase their macromolecule content (largely protein) to a point necessary to perform their specialized functions. This involves coordination between extracellular cues from nutrients and growth factors and intracellular signaling networks responsible for controlling cellular energy availability and macromolecular synthesis.

Perhaps the most tightly regulated cell growth occurs in dividing cells, where cell growth and cell division are clearly separable processes. Dividing cells generally must increase in size with each passage through the cell division cycle to ensure that a consistent average cell size is maintained. (There are examples in the animal kingdom where cell division in the absence of growth serves an important evolutionary function, such as during the syncytial division stage of the early developing *Drosophila* embryo.) For a typical dividing mammalian cell, growth occurs in the G_1 phase of the cell cycle and is tightly coordinated with S phase (DNA synthesis) and M phase (mitosis). The combined influence of growth factors, hormones, and nutrient availability provides the external cues for cells to grow. It is hypothesized that once dividing cells reach a threshold size, cells irreversibly commit to at least one round of division; achieving adequate size is thus a prerequisite for DNA synthesis and mitosis. Deprivation of nutrients and other growth signals, as might be the case in the nutrient (and oxygen)-starved regions of a growing tumor, may encourage normal cells to exit the cell cycle into a resting or G_0 state. Therefore, mutations in signaling pathways that promote growth independently of growth factors and nutrient availability may provide tumor cells with a selective growth advantage. Efforts to identify intracellular signaling networks that control growth are therefore a mainstay of many cancer-focused research programs.

Biochemical Pathways That Control Cell Growth

The mTORC1 Pathway

Essential to connecting cell growth control with cancer pathogenesis was the identification of intracellular signaling molecules that coordinate signals from nutrient availability, growth factors, and hormones with autonomous cell growth. In cells, these inputs converge upon a Ser/Thr protein kinase called *TOR* (target of rapamycin), which has emerged as a central controller of eukaryotic cell growth.[2] TOR was discovered in the 1970s to be the molecular target of an antifungal macrolide produced by a soil bacterium *(Streptomyces hygroscopicus)* that was isolated on Easter Island (Rapa Nui). Today rapamycin is recognized for its immunosuppressive function, ability to prevent restenosis after angioplasty, and limited anticancer properties. Mechanistically, rapamycin binds an intracellular protein called FKBP12 (an immunophilin), and the rapamycin-FKBP12 complex binds potently and specifically to TOR. Extensive studies across multiple eukaryotic model systems, all spawned by the identification of TOR as the target of rapamycin, have unveiled a complex TOR-centric signaling network responsible for integrating numerous growth signals into a metabolic program that drives biomass production (i.e., cell growth).

About a decade after the discovery of TOR, biochemical studies revealed that TOR (named *mTOR* in mammals in which the *m* now officially denotes "*m*echanistic" TOR) associates into at least two distinct multisubunit protein complexes called *mTOR complex 1* (mTORC1) and *mTORC2*. Growth control by mTOR is largely attributed to the best understood mTOR complex—mTORC1. In addition to the catalytic mTOR subunit, mTORC1 contains Raptor (regulatory associated protein of mammalian target of rapamycin), PRAS40 (proline-rich AKT substrate 40 kDa), mLST8 (mammalian lethal with sec-13 protein), and DEPTOR (DEP domain containing mTOR interacting protein) (Figure 12-1). Of these interacting proteins, Raptor and PRAS40 are unique to mTORC1, whereas mLST8 and DEPTOR are shared with mTORC2 (discussed later). Raptor is both a regulator of mTORC1 catalytic activity and a scaffold for recruiting mTORC1 substrates. PRAS40 and DEPTOR inhibit mTORC1 activity by undefined mechanisms. The function of mLST8 is unknown, but it does not appear to be required for mTORC1 activity. Rapamycin-FKBP12 directly binds mTORC1 through a domain in the mTOR catalytic subunit called the *FRB domain* (*F*KBP12-*r*apamycin-*b*inding domain), and although the drug destabilizes the association between mTOR and Raptor, it does not dissociate any components of the complex. In fact, rapamycin's exact mechanism of action remains a mystery despite years of research.

It is now widely accepted that mTORC1 positively controls an array of cellular processes critical for growth, including protein synthesis, ribosome biogenesis, and metabolism, and negatively influences catabolic processes such as autophagy—all of which have roles in cancer pathogenesis. Elucidating the key downstream targets of mTORC1 driving these events is an intense area of research. Originally, much of the study of mTOR relied on experiments in which rapamycin was used acutely to inhibit mTOR (which we now know was mTORC1) in cultured cells. This led to extensive characterization of the best known mTORC1 substrates eiF-4E-binding protein 1(4E-BP1) and S6 kinase 1 (S6K1), both of which regulate protein synthesis.[3] In the unphosphorylated state, 4E-BP1 binds and inhibits the cap-binding protein and translational regulator eIF4E. When phosphorylated by mTOR, 4E-BP1 is relieved of its inhibitory duty, promoting eIF4E interaction with the eIF4F complex and the translation of capped nuclear transcribed mRNA. Following co-regulatory phosphorylation by mTORC1 and another kinase called *phosphatidylinositol 3-dependent kinase 1* (PDK1), S6K1 positively affects mRNA synthesis at multiple steps including initiation and elongation by phosphorylating several translational regulators. Although the preponderance of evidence indicates that S6K1 and 4E-BP1 are directly phosphorylated by mTOR, an unidentified phosphatase activity may also be involved in their regulation. For example, the rapamycin-sensitive phosphorylation site on S6K1 is rapidly dephosphorylated (i.e., within minutes) of exposure to the drug.

For many years studies using rapamycin were at odds with the model that mTOR is an essential controller of protein synthesis because rapamycin has only modest effects on translation in most mammalian cells. Rapamycin is an allosteric inhibitor that binds mTOR outside of the kinase domain; it was therefore suspected that rapamycin might incompletely inhibit mTORC1. In 2009, the first mTOR kinase inhibitors became available (discussed later), which, unlike rapamycin, are ATP-competitive inhibitors that target the ATP-binding pocket of mTOR.[4,5] The first studies employing mTOR catalytic site inhibitors confirmed what had previously been suspected: that rapamycin only partially inhibits mTORC1 activity. The mTOR catalytic site inhibitors exposed rapamycin-resistant functions of mTORC1 in translational control, and consequently the mTOR ATP-competitive inhibitors have more profound effects on mRNA translation and cell proliferation than does rapamycin. Researchers are now using these new-generation mTOR inhibitors to identify novel mTORC1 substrates (many of which are rapamycin resistant) and beginning to fill in the gaps between mTOR and the myriad cellular processes it regulates.[6,7]

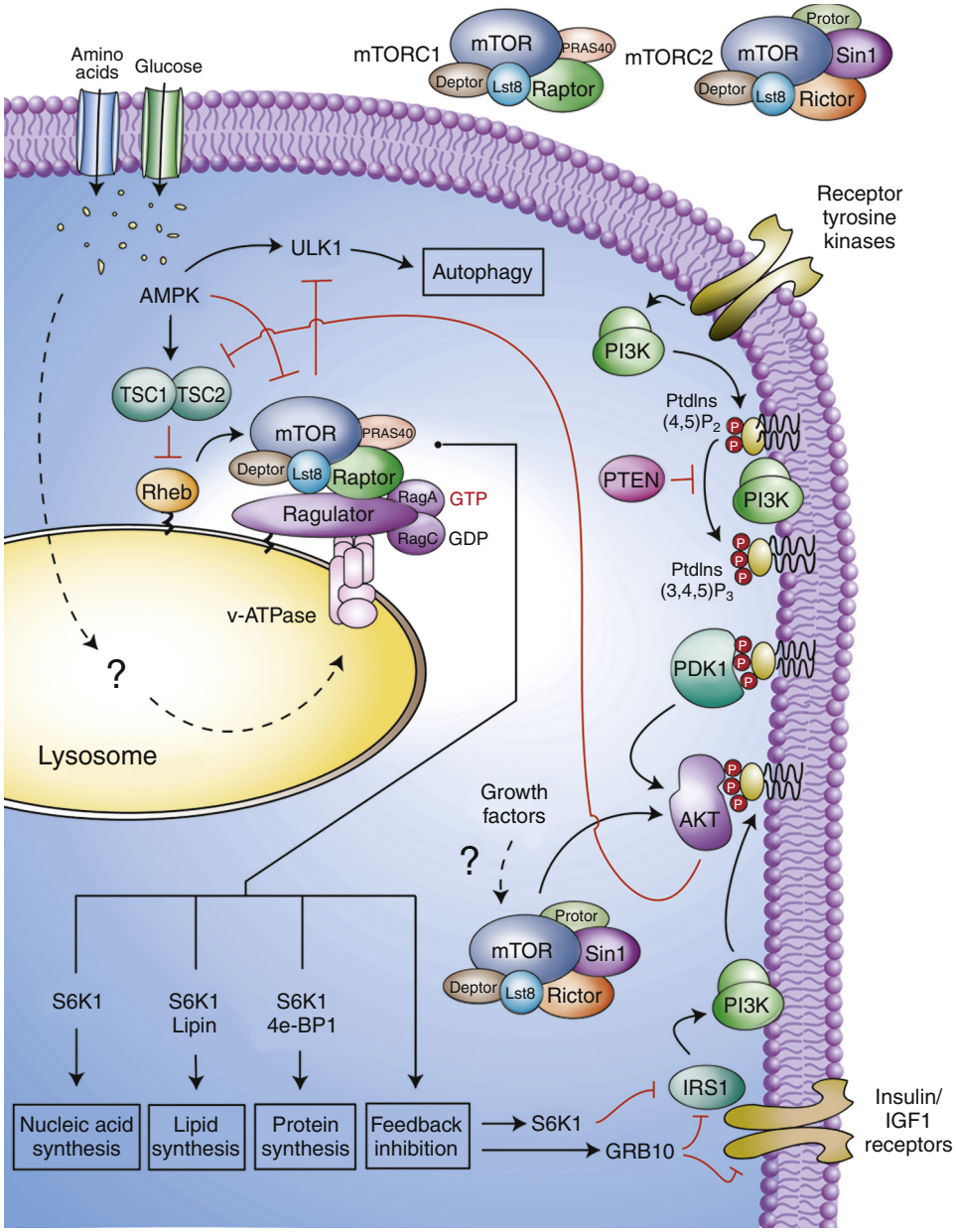


Figure 12-1 A simplified model of mTOR signaling in the active (i.e., nutrient-replete) state mTOR is the catalytic subunit of two distinct multisubunit complexes called mTORC1 and mTORC2 (described in the text). In nutrient-replete cells, the RAG-GTPase and Ragulator recruit mTORC1 to the lysosome, where it can be activated by the Rheb-GTPase. The RAG complex is active when RAGA or RABB (not shown) is in the GTP bound state and RAGC or RAGD (not shown) is bound to GDP. The active state is determined by an unknown amino acid- and glucose-sensing mechanism that exists inside the lysosome that signals through the v-ATPase to the Ragulator. The Ragulator possess guanine nucleotide exchange factor (GEF) activity toward RAGA/B and thereby triggers the switch that generates the mTORC1 lysosomal recruitment signal. The activation state of Rheb is controlled by the TSC complex, which senses energy availability (through AMPK) and growth factors (through AKT). In addition, AMPK can directly inhibit mTORC1 by phosphorylating Raptor, while AKT can activate mTORC1 by phosphorylating PRAS40 (not shown). When growth factors, energy, and nutrients are available, mTORC1 promotes anabolic processes such as protein, lipid, and nucleic acid biosynthesis and suppresses catabolic processes such as autophagy. mTORC1 activation also promotes feedback inhibition of growth factor signaling to maintain cellular homeostasis. In contrast to mTORC1, mTORC2 is poorly understood. The best-described mTORC2 function is to phosphorylate and fully activate AKT, suggesting that mTORC2 might also function upstream in controlling cell growth. However, AKT has many additional substrates that influence cell proliferation, cell survival, cell metabolism, and numerous other processes (not shown). Thus mTORC2 may have important roles in many other cancer-relevant pathways. *Black lines* represent positive regulatory signals; *red lines* are inhibitory signals. *Dotted lines* indicate undefined mechanisms.

In addition to synthesizing protein, growing and dividing cells need to synthesize lipids (to build the plasma membrane and the membranes of intracellular organelles) and nucleic acids (to make RNA and DNA). There is growing evidence that mTORC1 regulates lipid metabolism (and storage in the case of adipocytes) through SREBP1.[8] SREBPs are transcription factors that facilitate the expression of lipid and sterol biosynthesis enzymes. Several recent studies suggest that mTORC1 is an upstream regulator of SREBP1 activation. In addition

to driving lipid metabolism genes, SREBP1 can also increase the expression of genes involved in the pentose phosphate pathway, which produces important metabolites needed for both lipogenesis and nucleotide biosynthesis. How mTORC1 activates SREBP1 is still unclear, although it appears to function through multiple substrates including S6K1 and another direct mTORC1 substrate called Lipin1. In an mTORC1-dependent manner, S6K1 also controls pyrimidine biosynthesis by directly phosphorylating and regulating the activity of CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase), which catalyzes the first three steps of de novo pyrimidine synthesis.[9,10] Thus, mTORC1 truly is a master controller of growth, as it significantly influences the intracellular synthesis of the major macromolecules required by growing (and dividing) cells: proteins, lipids, and nucleic acids.

Upstream Signaling to mTORC1

The connection between cell growth control by mTORC1 and cancer solidified with the identification of upstream mTORC1 regulators.[2,11,12] Building biomass requires adequate building material, sufficient energy, and favorable environmental conditions. Therefore, it is not surprising that mTORC1 activity is controlled by numerous factors such as amino acid and glucose availability, ATP level, mitochondrial activity, growth factor signaling, and oxygen levels, all of which affect cancer cell growth. The discovery that the TSC1 (hamartin) and TSC2 (tuberin) tumor suppressors function together in a complex (the TSC complex) to negatively regulate mTORC1 provided a key first step in unraveling the biochemical mechanism of how upstream signals control mTORC1 activity. The TSC complex contains GTPase-activating protein (GAP) activity, which suppresses the activity of a small GTPase called *Rheb*. Rheb directly activates mTORC1 by an unknown mechanism, and therefore by promoting Rheb-GTP hydrolysis, the TSC complex suppresses mTORC1 (see Figure 12-1). Mutation in either the *TSC1* or *TSC2* gene results in aberrant upregulation of mTORC1 activity and causes a tumor-prone syndrome called *tuberous sclerosis complex* (described later).

The TSC complex integrates many positive and negative signals responsible for modulating mTORC1 activity. For example, extracellular growth factors such as insulin and insulin growth factor 1 (IGF-1) can activate an intracellular signaling pathway that inhibits TSC2. Through activation of receptor tyrosine kinases, growth factors stimulate PI3K to phosphorylate membrane-associated phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to generate phosphatidylinositol 3,4,5-triphosphate PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 serves as a docking site for the membrane recruitment and activation of the AKT kinase, one of the most versatile kinases in the human kinome. Among its many functions, AKT phosphorylates and inhibits TSC2 and thereby potentiates mTORC1 signaling.[13] AKT can also directly phosphorylate the PRAS40 subunit of mTORC1, which relieves a negative effect of this subunit on mTORC1 activity. The phosphatase and tumor suppressor PTEN, a major tumor suppressor in human cancer, balances PI3K activity by dephosphorylating PtdIns(3,4,5)P_3 and thus negatively regulates AKT activity. Other growth factor pathways such as the MAPK/ERK pathway and WNT pathway can also activate mTORC1 by inhibiting TSC as can proinflammatory cytokines such as tumor necrosis factor-α (TNFα) through the IκB kinase β (IKKβ).

Although extracellular growth factors provide the external (systemic) cues for growth, intracellular energy, oxygen, and nutrients must also be available.[2,11,12] An intracellular ratio of ATP to ADP and AMP is sensed by the AMP-activated protein kinase (AMPK). Under conditions of energy stress, high levels of ADP/AMP bind and activate AMPK, which suppresses anabolic processes and stimulates catabolic reactions. Active AMPK directly phosphorylates and activates TSC2 to suppress mTORC1 signaling when energy supplies drop. AMPK also directly inhibits mTORC1 by phosphorylating the Raptor subunit, emphasizing a theme that positive and negative regulators of growth often impinge on the mTORC1 pathway at multiple steps. Oxygen stress (i.e., hypoxia) inactivates mTORC1 through TSC, but by a different mechanism. In hypoxic conditions, hypoxia-inducible factor α (HIF1α) is stabilized, and its accumulation leads to a transcriptional response that promotes a metabolic shift away from oxidative phosphorylation toward oxygen-independent glycolysis, increased vascularization, and decreased mTORC1 activity by inducing expression of REDD1 and REDD2. REDD1/2 release TSC2 from an inhibitory interaction with 14-3-3 proteins. Notably, mTORC1 can also potentiate HIF1α by increasing its transcription and translation, possibly functioning as part of a feedback regulatory circuit. Finally, DNA damage can reduce mTORC1 activity in part by increasing expression of TSC2 and PTEN.

Nutrient Regulation of mTORC1

Amino acids signal mTORC1 activation independently of growth factors. In fact, amino acids may be the most critical activator of mTORC1, as their ability to stimulate TOR activity is conserved from yeast to human. However, elucidating the mechanism of amino acid regulation has lagged behind many of the other upstream signals because, unlike the other inputs, amino acids do not activate mTORC1 through the TSC-Rheb

axis. However, the mechanism behind amino acid regulation of mTORC1 is now beginning to be revealed.[14,15] The first major leap forward in unraveling the mystery of how amino acids regulate mTORC1 was the discovery in mammals of four small GTPases called the *RAG* GTPases (i.e., RAGA, RAGB, RAGC, RAGD). RAGA or RAGB (which are structurally similar to each other and functionally redundant) form heterodimers with RAGC and RAGD (which are also similar to each other and functionally redundant). In the presence of amino acids, RAGA/B are GTP bound, whereas RAGC/D are GDP bound. When starved of amino acids, the situation is reversed: RAGA/B are GDP bound and RAGC/D are bound to GTP. Active RAG heterodimers bind directly to mTORC1 through the Raptor subunit. Unexpectedly, the RAG GTPases do not directly activate mTORC1 activity, but rather redistribute mTORC1 from an unknown location in the cytoplasm to the lysosome. The RAGs are associated with the lysosome through interaction with a pentameric complex called the *Ragulator* (which contains the p18/LAMTOR1, p14/LAMTOR2, MP1/LAMTOR3, C7orf59/LAMTOR4, and HBXIP/LAMTOR5 subunits). The Ragulator is tethered to the lysosome through the p18/LAMTOR1 subunit, which is both myristoylated and palmitoylated. In addition, the Ragulator contains guanine nucleotide exchange factor (GEF) activity toward RAGA/B, indicating that it is also functionally involved in activating the RAG recruitment signal. Thus, when amino acids are present, the Ragulator loads RAGA/B with GTP, triggering mTORC1 recruitment to the lysosome, where it docks with the RAG/Ragulator. The mechanism that delivers mTORC1 to the lysosome remains unknown.

How amino acids signal to the Ragulator to activate the RAG recruitment signal is also unclear. Early work suggests that amino acids might actually signal mTORC1 recruitment to the lysosome from within the lysosome by an "inside-out" mechanism. This mechanism involves the vacuolar H^+-ATPase (v-ATPase), which senses intralysosomal amino acids by an unknown mechanism and relays that information to the Ragulator through an amino acid–sensitive direct interaction. The v-ATPase is composed of many individual protein subunits and hydrolyzes ATP to pump protons into the lysosome to acidify the lumen. The v-ATPase is required for amino acids to trigger RAG-dependent recruitment of mTORC1, but it is not required for the RAG GTPases to associate with the Ragulator. The exact pool of amino acids being delivered to the lysosome remains to be determined. Thus, the v-ATPase appears to function downstream of amino acids but upstream of the RAG GTPases by promoting Ragulator GEF activity and therefore RAGA/B GTP loading.

Why does mTORC1 need to localize to the lysosome? Because the RAG GTPases do not activate mTORC1, it is hypothesized that mTORC1 must be recruited to the lysosome to be activated. In line with this idea, Rheb is proposed to reside on endomembranes including the lysosome, and thus mTORC1 recruitment would bring the complex in close proximity to its activator. This model explains why mTORC1 cannot be activated by growth factors in the absence of amino acids: that is, growth factors activate lysosomally tethered Rheb, but in order for Rheb to promote growth, mTORC1 must be delivered to the lysosome, which requires amino acids. In fact, forcing mTORC1 and Rheb to interact by overexpressing Rheb, artificially tethering mTORC1 to the lysosome, or artificially tethering both mTORC1 and Rheb to the plasma membrane results in amino acid–independent mTORC1 activation. More recent work suggests glucose may also mediate RAG-dependent recruitment of mTORC1 to the lysosome.[16] Like amino acids, glucose stimulates interaction between the v-ATPase and the Ragulator, suggesting that amino acids and glucose availability may converge on a common mechanism of mTORC1 regulation inside the lysosome.

Whether additional amino acid–dependent mechanisms of mTORC1 regulation exist is an open question. Although the Rag-Ragulator mechanism controls mTORC1 localization and activation in response to general amino acid and glucose availability, there could be additional inputs from mechanisms in the cytoplasm that sense specific amino acids. For example, it has been proposed the leucyl-tRNA synthetase (which catalyzes the ligation of L-Leu to its cognate tRNA) can act as a direct sensor of intracellular leucine and in turn promote mTORC1 activation. The exact mechanism and significance of this input remain to be determined. Nevertheless, these emerging studies indicate that intricate mechanisms evolved to link mTORC1 growth signaling to nutrient availability—independently of growth factors—ensuring that growth does not occur unless the building materials and energy supply are sufficient to meet the metabolic demands of macromolecular biosynthesis.

PI3K-AKT Signaling, mTORC1, and Cancer

Conditions that inhibit growth, such as decreased energy, low oxygen, and insufficient nutrients, are associated with the harsh microenvironment of poorly vascularized tumor. The ability of cancer cells to overcome these adverse conditions would promote tumor growth, putting the desensitization of mTORC1 signaling in the spotlight as a potential mechanism cancer cells could exploit to enhance their viability. Whether mutations in the amino acid– and glucose-sensing pathway that activates mTORC1 exist in cancer is not known. Mutations in the growth factor inputs to mTORC1 are prominent in cancer. For example, mutations causing loss of PTEN function or oncogenic activation of

PI3K or AKT are associated with many aggressive human cancers (Table 12-1).[17-20] The findings that AKT promotes mTORC1 activity through TSC and PRAS40 suggest that cancers with elevated PI3K-AKT signaling may in part thrive because of an mTORC1-driven growth advantage. Activation of PI3K-AKT signaling also facilitates nutrient uptake by cells, which indirectly contributes to mTORC1 activity by localizing mTORC1 to lysosomes. Therefore, understanding the contribution and relevance of mTORC1 signaling in the progression of cancers with aberrant PI3K-AKT signaling is an important area of research.

Unlike tumor cells with PTEN loss, tumor cells that have lost TSC function are generally less belligerent. It is now accepted that this is due to negative feedback loops that suppress the PI3K-AKT pathway when mTORC1 activity is high.[2,6,7] For example, the mTORC1 substrate S6K1 can directly phosphorylate and inactivate the IRS1 and IRS2 proteins, two mediators of PI3K activation, which decreases PI3K-AKT activity. A second feedback mechanism occurs through the direct mTORC1 substrate Grb10, which is activated by mTORC1 and functions to inhibit both the insulin/IGF receptors and IRS proteins. Negative feedback mechanisms would normally keep cell growth and nutrient uptake in balance, but in the absence of TSC, negative feedback squelches PI3K and AKT activity, which is important for promoting cell survival and metastasis. This may be why tuberous sclerosis (discussed later), which is caused by mutations in TSC, generally does not develop into metastatic cancer. Because PTEN and PI3K are downstream of the insulin/IGF receptors and IRS proteins, cancers driven by loss of PTEN or PI3K activation are uncoupled from these particular mTORC1-driven negative feedback loops, explaining why they are typically associated with more aggressive cancer.

Table 12-1 mTOR Signaling in Disease

Disease	Linked Genetic Mutation and Clinical Pathology	Predicted Functional Link to mTOR Signaling
Tumor-prone Syndromes		
TSC (tuberous sclerosis complex)	*TSC1* or *TSC2;* hamartomas in multiple organs	TSC1/2 negatively regulates Rheb
LAM (lymphangioleiomyomatosis)	*TSC2;* abnormal proliferation of smooth-muscle–like cells in the lung	TSC1/2 negatively regulates Rheb
Cowden disease	*PTEN;* hamartomatous tumor syndrome	May promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
Proteus syndrome	*PTEN;* hamartomatous tumor syndrome	May promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
Lhermitte-Duclos disease	*PTEN;* hamartomatous tumor syndrome	May promote AKT-dependent inhibition of TSC2 and mTOR phosphorylation
PJS (Peutz-Jeghers syndrome)	*STK11/LKB1;* gastrointestinal hamartoma tumor syndrome	STK11 activates AMPK, a positive regulator TSC2
HCM (familial hypertrophic cardiomyopathy)	*AMPK;* myocardial hypertrophy	AMPK promotes TSC2 function
Cancer		
Prostate	*PTEN*	PTEN loss promotes AKT activation
Breast	*PTEN;* PI3K, *AKT,* or *Her2/neu* amplification/ hyperactivation	PTEN loss or gene amplifications promote AKT activation
Lung	*PTEN; HER* amplification	PTEN loss or gene amplifications promote AKT activation
Bladder	*PTEN*	Promotes AKT activation
Melanoma	*PTEN*	Promotes AKT activation
Renal-cell carcinoma	*PTEN*	Promotes AKT activation
Ovarian	*PTEN;* PI3K, *AKT,* or *Her2/neu* amplification/ hyperactivation	PTEN loss or gene amplifications promote AKT activation
Endometrial	*PTEN*	Promotes AKT activation
Thyroid	*PTEN;* PI3K, *AKT,* or *Her2/neu* amplification/ hyperactivation	PTEN loss or gene amplifications promote AKT activation
Brain (glioblastoma)	*PTEN*	Promotes AKT activation
CML (chronic myeloid leukemia)	*BCR/ABL* translocation	Promotes AKT activation

mTORC1 and Autophagy

Macroautophagy, or simply autophagy, is a catabolic process induced on nutrient limitation or stress in which autophagosomes form and fuse with the lysosome to create an autophagolysosome. The autophagolysosome is the final destination for proteins and organelles that are broken down into their basic components to be recycled as sources of cellular energy and metabolites during starvation. Autophagy has an important role in cell survival during times of energy crisis. Under normal growth conditions, autophagy also salvages and recycles cellular junk such as excess or damaged organelles. Newborn mice require autophagy to survive a brief starvation period immediately after birth, and defects in autophagy are associated with cancer, degenerative disorders, and aging.[21-23] A connection between mTORC1 signaling at autophagy first became apparent in yeast and mammalian cells in studies where rapamycin was found to induce autophagy. Genetic inactivation of TOR in *Drosophila* also induces autophagy and confirmed that mTORC1 not only positively controls anabolic processes, but suppresses catabolic processes opposing growth.

Recent studies have revealed that the regulation of autophagy by mTORC1 is at least partly direct and occurs in coordination with AMPK.[24,25] More than 30 autophagy *(ATG)* genes have been identified that control autophagy. In nutrient-rich conditions, mTORC1 associates with and directly phosphorylates ULK1/ATG1, the kinase subunit of a complex that is required for autophagy induction. Phosphorylation of ULK1 by mTORC1 inhibits its activity and reduces autophagic vesicle formation. In nutrient-deprived cells, AMPK not only inhibits mTORC1 by phosphorylating TSC2 and Raptor (as described earlier), but also directly activates ULK1 kinase activity. When mTORC1 activity decreases, ULK1 is dephosphorylated, allowing AMPK to now interact with, phosphorylate, and activate ULK1 to induce autophagy. This dual regulatory circuit emphasizes the tight balance between anabolic and catabolic processes.

Interestingly, prolonged autophagy, which delivers its cargo to the lysosome, eventually reactivates mTORC1. Thus, in nutrient-starved cells, autophagy may also be required to keep mTORC1 on in a minimally activated state to promote the synthesis of certain macromolecules essential for survival. The reactivation of mTORC1 also promotes the formation of nascent lysosomes that bud from the autophagosome. In fact, recent studies further suggest that mTORC1 may normally regulate lysosome formation through its ability to control that nuclear localization (and thus activity) of a transcription factor called *TFEB*. TFEB regulates the expression of many genes important for lysosome formation. Thus, the connections among nutrient and energy sensing, mTORC1 growth control, and protein/organelle turnover by autophagy and the lysosome are complex, and it will be interesting to determine if and how defects in these connections promote cancer.

Although the connections between mTORC1 and autophagy are being worked out, the strong connections between mTORC1 and cancer suggest that autophagy may have a role in tumorigenesis, and evidence supports this hypothesis.[21] For example, mice deficient for a pro-autophagy gene, called *beclin-1*, have an increased frequency of spontaneous tumor formation, suggesting that *beclin-1* is a tumor suppressor gene. How could inactivation of autophagy promote tumorigenesis? It is difficult to speculate at this time because little is known about Beclin 1, but perhaps some transformed cells rely on autophagy to remove damaged organelles or for complete self-disposal. In this model, mutations that prevent autophagy would promote cell survival. Interestingly, the Bcl-2 pro-survival oncoprotein, which has long been thought to inhibit apoptosis, binds and inhibits Beclin 1–dependent autophagy.[26] This suggests that the oncogenic activity of Bcl-2 might be linked to inhibiting nonapoptotic autophagic cell death. Contrary to having tumor suppressor capacity, autophagy could promote tumorigenesis in some cases. When cancer cells experience nutrient limitation, autophagy may provide the rations necessary to sustain the most essential bioenergetic cellular process. This may allow cancer cells to survive until angiogenesis is initiated or other deleterious mutations occur. Understanding the role of autophagy in different cancer cells will be important to designing mTOR-focused treatment strategies.

mTORC1 and p53

Mutation of the p53 tumor suppressor is one of the most common genetic abnormalities associated with human cancer. DNA or spindle damage, telomere shortening, various stresses, or oncogenic mutations will usually initiate a p53-dependent checkpoint that arrests cells in G_1 or trigger apoptosis (depending on the cell type and signals present). This has led to speculation that such a checkpoint mechanism involving p53 might communicate with the mTORC1 pathway to discourage growth when conditions are unfavorable. Early reports from work with cultured cells suggest that AMPK and p53 may communicate in some type of checkpoint mechanism to restrict growth in low-glucose conditions or when DNA is damaged, although it is unclear from this early work if and how directly mTORC1 regulation might be connected.[27] Mounting evidence also indicates that a communication link exists between p53 and the regulation of mitochondrial respiration, particularly in mediating a cellular switch to preferentially deriving energy from glycolysis rather than aerobic respiration—a hallmark characteristic of cancer cells.[28] It is well known that p53 and

the mitochondria, and to some extent mTOR, have roles in apoptosis. Because signals from the mitochondria are also thought to control mTORC1's ability to mediate growth, it seems likely that a complicated signaling circuitry involving both the p53- and mTOR-dependent pathway exists.

Beyond Cell Growth: Does mTOR Regulate Organ and Organism Growth?

The mTORC2 Pathway

The role of TOR in building cell mass is well documented and conserved in all eukaryotic organisms examined. However, extending a role for mTOR in controlling organ and organism growth is more complicated, because most tissues grow by mechanisms involving the collective coordination of cell growth, cell division, and cell death pathways. The finding that mTOR exists in a second complex called *mTORC2* does suggest that mTOR may have broad influence over cell growth, cell division, cell death, metabolism, and many other processes essential for building tissues and organisms. Many of these additional targets of mTOR activity, although not completely worked out yet, are likely to be important in cancer progression, and thus emerging therapeutic strategies are now aiming to more broadly target mTOR signaling by inhibiting both mTORC1 and mTORC2.

The second mTOR complex also contains mLST8 and Deptor, but instead of Raptor and PRAS40, mTORC2 contains the Rictor, mSin1, and PROTOR proteins (see Figure 12-1).[2] Understanding the function of mTORC2 initially eluded researchers because the complex is insensitive to acute rapamycin treatment, and thus rapamycin could not be used to probe for mTORC2 substrates. However, advances in RNA-interference technology allowed the specific depletion of Rictor from cells, leading to the discovery that mTOR, when outfitted with mTORC2-specific regulatory proteins, phosphorylates and activates the AKT kinase. Current models suggest that on recruitment to the membrane after growth factor stimulation, AKT is phosphorylated by mTORC2 in conjunction with PDK1, and this co-regulation is presumed necessary for full AKT activation. How mTORC2 activity itself is controlled remains to be worked out, but initial experiments indicate that growth factors also modulate mTORC2 activity by an unknown mechanism.

AKT activity influences cell growth, cell proliferation, cell survival, metabolism, and many other processes.[13] Thus, by coupling mTOR activity with proliferation and survival, the discovery of mTORC2, together with the well-known role of mTORC1 in controlling cell size, strengthens the argument that mTOR growth regulation extends beyond cell autonomous growth to organ and organism growth. Moreover, because of the widespread role of PI3K-AKT signaling in cancer (see Table 12-1), the finding that mTORC2 phosphorylates and activates AKT is a compelling additional link between mTOR and cancer. In fact, a number of studies now suggest that selectively targeting mTORC2 could also be a promising therapeutic strategy.[29,30] What is interesting about potentially targeting mTORC2 is that in many cases, loss of mTORC2 function (by genetic approaches) is less toxic to normal cells than targeting mTORC1 function. In contrast, mTORC2 activity is required for transformation, particularly when transformation is driven by loss of PTEN.[31] Thus, mTORC2 might be more essential to certain cancer cells than to many normal cells. The challenge of selectively targeting mTORC2 is that beyond the mTOR catalytic domain, the understanding of mTORC2 structure and regulation is only vague, and thus it is currently difficult to predict what type of targeting strategy might best achieve this goal.

In retrospect, it is not surprising that mTOR can also phosphorylate AKT, because the mTORC2 phosphorylation site on AKT (S473) is structurally similar to the mTORC1 site on S6K1 (T389), both of which are present in carboxy-terminal hydrophobic motifs. In fact, both S6K and AKT belong to a larger family of structurally related kinases called the AGC kinases.[32] The discovery of the mTORC2-AKT signaling module also sets up the peculiar situation whereby mTOR can regulate itself in a manner dependent on what proteins it interacts with. For instance, mTORC2-mediated phosphorylation of AKT is proposed to promote subsequent phosphorylation and inactivation of TSC2 and PRAS40 (as described earlier), which would place mTORC2 upstream of mTORC1 regulation. The extent to which normal and cancer cells rely on such regulation is under investigation. How other AKT substrates are regulated by mTORC2 is also largely unclear. In addition to AKT, mTORC2 can directly phosphorylate two additional AGC kinases called SGK and PKC. SGK is most similar to AKT, except that it lacks the membrane-targeting PH domain that brings AKT to its activation site at the cell membrane. SGK also has some overlapping functions with AKT, but currently the role of mTORC2-mediated SGK and PKC regulation in cancer is unknown.

Controlling Body Size

It is a remarkable feat for mammals to coordinate the development of their organs to appropriate sizes that are proportional to overall body size. The endocrine system is responsible for conducting this massively orchestrated systemic growth. The major hormone responsible for controlling postnatal growth is growth hormone (GH). GH is synthesized in the pituitary gland. After its release into the blood, circulating GH stimulates the release of insulin-like

growth factors (IGFs) from the liver. IGFs subsequently stimulate growth of bone and muscle, which are the two organs most relevant to determining body size.

The findings (1) that mTOR regulates cell growth, proliferation, and survival and (2) that both mTORC1 and mTORC2 are downstream of insulin/IGF signaling raise the question of whether mTOR is a master regulator of body size. The role of mTOR as a nutrient-sensitive regulator of eukaryotic cell growth (i.e., as part of mTORC1) is an ancient function conserved from yeast to humans. During the evolution of multicellular organisms, growth factor signaling was grafted onto the mTORC1 pathway, probably to modulate autonomous cell growth in conjunction with systemic nutrient availability and signals from other cells and tissues. The discovery that mTOR (as part of mTORC2) regulates AKT further suggests that additional pathways independent of mTORC1 may contribute to the regulation of cell growth, proliferation, survival, and metabolism. Many mTORC2 components are also conserved in yeast—although the protein sequences and downstream functions are more diverged compared to mTORC1, suggesting the cellular functions of mTORC2 may have also diverged. Because of mTOR's remarkable ability to integrate numerous signals and control many aspects of cell growth, it seems poised to be a master regulator of organ and body size control. Studying this in mammals has been difficult, however, because mice null for mTOR and the essential mTORC1 and mTORC2 regulatory subunits are embryonic lethal. In *Drosophila*, the fat body (which may be equivalent to the vertebrate liver) functions as a nutrient-sensing organ that can control body growth through a TOR-dependent mechanism.[33] Moreover, mice and flies deficient for S6K1 are viable but reduced in size because of smaller cells, implying a link between mTORC1 signaling and body size control.[34] Thus, limited evidence in model organisms suggests that mTOR could influence body size by systemic as well as cell-autonomous mechanisms. Interestingly, in humans a rare mutation in the p14/LAMTOR2 subunit of the Ragulator delays growth and causes immunosuppression and hypopigmentation.[35] As novel upstream regulators and substrates of mTOR are unveiled, it will be interesting to determine if genetic variation in components of the mTOR network has a role in determining organ and organism size.

Targeting mTOR Signaling as a Treatment for Cancer and Other Human Diseases of Cell Growth

With a role for mTOR signaling in cancer now firmly established, developing molecules that inhibit mTOR for therapeutic purposes is a significant focus of the pharmaceutical industry. As mentioned earlier, rapamycin (also known as *sirolimus*) is used clinically for immunosuppression (because of its ability to suppress T-cell proliferation), to prevent restenosis (by preventing the growth of vascular smooth muscle cells), and to treat certain cancers.[5,17,36] Analogs of rapamycin have also been developed for cancer therapy (CCI779/temsirolimus, AP23573, and RAD001/everolimus—collectively called rapalogues). Unfortunately, rapamycin as a broad anticancer drug has had limited success as a single-agent therapeutic, even against cancers with elevated PI3K-AKT signaling. The response to rapamycin is highly variable depending on the cancer, and only a few cancers, including mantle-cell lymphoma, renal-cell carcinoma, and endometrial cancers, have shown consistent positive responses. Temsirolimus was approved in 2007 to treat renal-cell carcinoma. Several trials are ongoing, although there is no clear biomarker predictive of rapamycin sensitivity.

A number of reasons have been proposed to explain rapamycin's general ineffectiveness as a cancer drug. One possibility (discussed earlier) is that inhibiting mTORC1 relieves strong negative feedback loops that function to suppress receptor tyrosine kinase (RTK) signaling and downstream PI3K-AKT activation (Figure 12-2). Therefore, treating cells with rapamycin boosts AKT signaling, which in turn could drive AKT-dependent cell proliferation and survival pathways—clearly an undesirable response. The MAPK-ERK signaling pathway is also triggered by mTORC1-S6K1 inhibition. Using rapamycin in combination with PI3K or MAPK pathway inhibitors might prove to be a better strategy. Second, it is now clear that rapamycin only partially inhibits mTORC1. For example, although rapamycin results in rapid dephosphorylation and sustained inactivation of S6K, phosphorylation of 4E-BP1 is only partially and transiently decreased.[37] Therefore, translation is largely insensitive to rapamycin. As more mTORC1 substrates are being discovered, it will be interesting to determine why substrates vary in their sensitivity to rapamycin.

It is often stated that rapamycin specifically targets mTORC1, implying that it does not inhibit mTORC2. However, the actual situation is more complex.[2,38] In cultured cells, rapamycin acutely affects only mTORC1, but prolonged exposure disrupts mTORC2 integrity, likely by blocking the assembly of new mTORC2 complexes. In some cell types this results in decreased AKT phosphorylation, but often there is no apparent change or increased AKT phosphorylation. Notably, any mTORC2 complexes that remain intact following rapamycin treatment are still susceptible to losing feedback inhibition. This likely explains the cell-to-cell variability in AKT phosphorylation following prolonged rapamycin treatment. For example, even if most mTORC2 complexes are

disrupted by rapamycin, what remains intact is hyperactive, and consequently AKT phosphorylation can appear insensitive to rapamycin or increase. Exactly what determines whether AKT phosphorylation in a particular cancer cell will show increased, decreased, or no sensitivity to rapamycin remains a mystery. Nevertheless, inhibition of mTORC2 following chronic exposure to rapamycin could explain the drug's effectiveness in certain cancers as well as some of its side effects. Dissecting the molecular mechanisms by which rapamycin functions and identifying biomarkers predictive of rapamycin sensitivity remains an important area of research.

Next-generation mTOR inhibitors are ATP competitive inhibitors (discussed earlier). Therefore, unlike the rapalogues (which are allosteric inhibitors), these drugs directly target mTOR kinase activity.[2,5] Consequently, the ATP competitive inhibitors more completely inhibit mTORC1 and also inhibit mTORC2. The mTOR ATP-competitive inhibitors are having a major impact in basic research, and several versions are being evaluated in clinical trials as anticancer drugs with the hope that they will outperform the rapalogues. Their tolerability remains to be seen, but early data are encouraging. Notably, losing feedback inhibition remains a challenge with these inhibitors because, despite the fact that they inhibit mTORC2 as well, the mTORC2 substrate AKT is also regulated by the PDK1 kinase, which increases activity following mTORC1 inhibition because the negative feedback is lost (see Figure 12-2). A related class of inhibitors is called the *dual-specificity inhibitors*, which target mTORC1, mTORC2, and PI3K. Dual inhibition is possible because the kinase domains of mTOR and PI3K are structurally similar, even though mTOR phosphorylates proteins and PI3K phosphorylates lipids. The dual-specificity inhibitors could have an advantage because by also inhibiting PI3K, they can mitigate the effects of losing the mTORC1-dependent negative feedback inhibition of PI3K-AKT signaling (see Figure 12-2). Because many

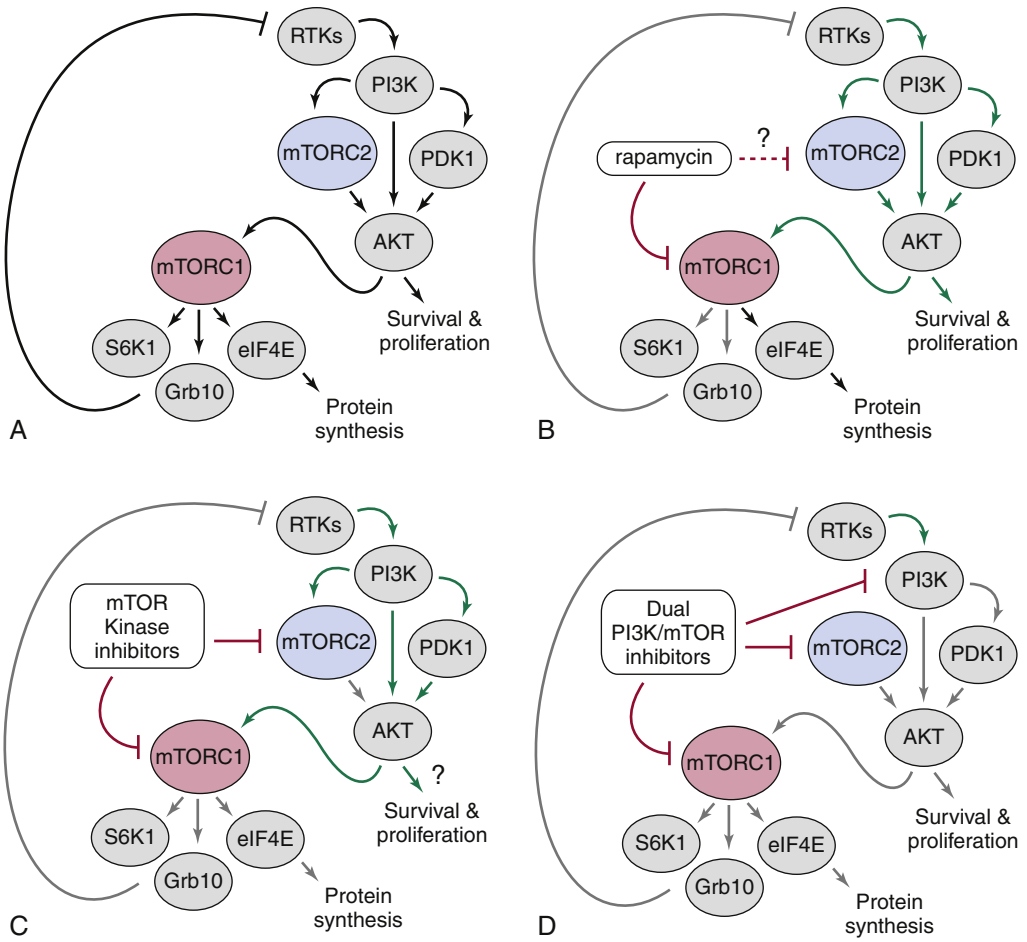


Figure 12-2 Model explaining how downstream signaling in cancer cells responds to currently available classes of mTOR pathway inhibitors **(A)** Simplified depiction of how the mTOR signaling circuitry is wired (described in the text). Note that PI3K is shown here activating mTORC2; however, exactly how growth factors activate mTORC2 is unknown. **(B)** Rapamycin (or the rapalogues) only partially inhibits mTORC1 and relieves strong mTORC1-dependent negative feedback inhibition of receptor tyrosine kinase (RTK)-PI3K signaling. Note that prolonged rapamycin treatment can inhibit mTORC2 in some cell types, but the therapeutic significance of this is unclear. **(C)** The mTOR kinase inhibitors are ATP-competitive inhibitors that target both complexes. However, these inhibitors also release PI3K from mTORC1-dependent feedback inhibition. **(D)** The dual-specificity inhibitors target both mTOR complexes and PI3K and thus may mitigate the consequences of losing feedback inhibition.

research studies and clinical trials are ongoing with both ATP-competitive and dual-specificity mTOR inhibitors, it should soon become clear to what extent they will be useful clinically.

Beyond the obvious rationale for targeting mTOR in human cancer, a variety of tumor-prone syndromes are also linked to mutations that impinge on mTOR (see Table 12-1).[18,39-42] These include tuberous sclerosis complex (caused by mutations in the *TSC1* or *TSC2* genes, a related disease called lymphangioleiomyomatosis or LAM (*TSC1* and *TSC2*), Cowden disease (*PTEN*), Bannayan-Riley-Ruvalcaba syndrome (*PTEN*), Proteus and Proteus-like syndrome (*PTEN*), Peutz-Jeghers syndrome (*LKB1*—an upstream activator of AMPK), and neurofibromatosis (*NF1*—a negative regulator of Ras-PI3K activity). Patients suffering from these syndromes are candidates for therapeutic intervention with mTOR inhibitors.

Tuberous sclerosis complex is characterized by benign tumors that can grow in the brain, kidney, heart, eyes, lungs, and skin and can be devastating and fatal depending on the degree of penetrance and location of the tumors. LAM usually affects women and results in abnormal growth of smooth muscle cells in the lungs that severely compromises respiration. The discovery that TSC negatively regulates mTORC1 propelled rapamycin into clinical trials to treat patients suffering from these diseases. Some features of tuberous sclerosis complex, such as the appearance of abnormally large astrocytomas in the brain, are reduced in size following rapamycin treatment, and everolimus is now FDA approved for treating this disease. Notably, the TSC complex may also have mTORC1-independent functions, and some studies have concluded that not all cellular phenotypes associated with losing TSC function are mTOR dependent. Although the rationale to use mTOR inhibitors to treat tuberous sclerosis complex and LAM is compelling, it will be important to consider the mTOR-independent roles of TSC when evaluating trial results.

Inactivation of PTEN can cause Cowden disease in addition to cancer. Cowden disease is characterized by hamartomas present in the skin, mucosa, gastrointestinal tract, bones, central nervous system, eyes, and genitourinary tract. Patients are at a high risk for breast and thyroid carcinoma. As described earlier, PTEN negatively regulates the PI3K-AKT signaling pathway, which can subsequently inactivate the TSC complex. Peutz-Jeghers syndrome is caused by inactivation of the LKB1 tumor suppressor and results in intestinal hamartomatous polyps and increased risk of intestinal cancer. LKB1 is a protein kinase that phosphorylates and activates AMPK. As discussed earlier, AMPK inactivates mTORC1 following energy depletion by promoting TSC function and by phosphorylating Raptor. Neurofibromatosis primarily results in tumors growing on nerve tissue but can also cause skin and bone abnormalities. The disease results from inactivation of NF1, a Ras-GTPase activating protein that, when absent, renders phosphorylation of TSC2 and S6K1 by AKT and mTORC1, respectively, resistant to growth factor (but not nutrient) withdrawal. Loss of NF1 leads to accumulation of Ras in the active GTP-bound state, suggesting that Ras, a well-known oncogene, also affects mTORC1 activity.

The links between signaling networks that control cell, organ, and organism growth through mTOR and the onset of human cancer suggest that therapeutic interventions targeting this pathway and its regulators could have promise. The potential for rapamycin and its analogs in treating cancer and other growth diseases captivated the pharmaceutical industry, and substantial investments in its development have led to extensive and ongoing clinical assessment. Despite years of research, rapamycin's mechanism of action remains enigmatic, and it is now clear that rapamycin only partially inhibits mTORC1. Effective use of the drug as an anticancer agent will require a deeper understanding of why only subsets of cancer cells are sensitive and whether there are efficacious combination therapies that can be exploited. Discovering that mTOR exists in at least two distinct complexes with differing sensitivity to rapamycin provided a strong rationale for developing novel inhibitors that target both complexes. Preclinical and clinical trials should soon inform the community of how efficacious these drugs will be. Other new potential avenues for developing mTORC1 pathway inhibitors include targeting the amino acid– and glucose-sensing pathway that delivers mTORC1 to the lysosome. However, it is possible that mTORC1 is so essential for maintaining normal cellular homeostasis that inhibiting it systemically will be too toxic to normal cells. Selectively inhibiting mTORC2 is an alternative strategy, at least in PI3K-driven cancers, as this could be less toxic to normal cells and leave mTORC1-dependent negative feedback loops largely intact. This, however, may prove to be a daunting task, as there is currently no obvious mTORC2 functional domain (outside the mTOR kinase domain) or essential upstream kinase that can be targeted. Because unique interacting proteins define the mTOR complexes, potential exists for developing mTORC2-selective inhibitors that might disrupt complex integrity or localization. Obtaining greater knowledge of the structure and function of the individual mTORC2 subunits will be essential for this endeavor. The discovery that prominent cancer pathways impinge on mTOR signaling has provided many new potential avenues for drug development, and ongoing efforts to develop mTOR inhibitors will undoubtedly affect the care of cancer patients.

Key References

2. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
3. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol*. 2009;10:307-318.
4. Liu Q, Thoreen C, Wang J, Sabatini D, Gray NS. mTOR mediated anti-cancer drug discovery. *Drug Discov Today Ther Strateg*. 2009;6:47-55.
5. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov*. 2011;10:868-880.
8. Ricoult SJ, Manning BD. The multifaceted role of mTORC1 in the control of lipid metabolism. *EMBO Rep*. 2013;14(3):242-251.
12. Yuan HX, Xiong Y, Guan KL. Nutrient sensing, metabolism, and cell growth control. *Mol Cell*. 2013;49:379-387.
13. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007;129:1261-1274.
17. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell*. 2007;12:9-22.
29. Sparks CA, Guertin DA. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. *Oncogene*. 2010;29:3733-3744.
36. Guertin DA, Sabatini DM. The pharmacology of mTOR inhibition. *Sci Signal*. 2009;2:pe24.
41. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006;124:471-484.

References

1. Guertin DA, Sabatini DM. Cell size control. *Encyclopedia of Life Sciences*. New York, NY: John Wiley & Sons, Ltd; 2005.
2. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
3. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol*. 2009;10:307-318.
4. Liu Q, Thoreen C, Wang J, Sabatini D, Gray NS. mTOR mediated anti-cancer drug discovery. *Drug Discov Today Ther Strateg*. 2009;6:47-55.
5. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov*. 2011;10:868-880.
6. Hsu PP, Kang SA, Rameseder J, et al. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science*. 2011;332:1317-1322.
7. Yu Y, Yoon S-O, Poulogiannis G, et al. Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science*. 2011;332:1322-1326.
8. Ricoult SJ, Manning BD. The multifaceted role of mTORC1 in the control of lipid metabolism. *EMBO Rep*. 2013;14(3):242-251.
9. Ben-Sahra I, Howell JJ, Asara JM, Manning BD. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science*. 2013;339:1323-1328.
10. Robitaille AM, Christen S, Shimobayashi M, et al. Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science*. 2013;339:1320-1323.
11. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science*. 2011;334:678-683.
12. Yuan HX, Xiong Y, Guan KL. Nutrient sensing, metabolism, and cell growth control. *Mol Cell*. 2013;49:379-387.
13. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007;129:1261-1274.
14. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. *Nat Rev Mol Cell Biol*. 2013;14:133-139.
15. Efeyan A, Zoncu R, Sabatini DM. Amino acids and mTORC1: from lysosomes to disease. *Trends Mol Med*. 2012;18:524-533.
16. Efeyan A, et al. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature*. 2013;493:679-683.
17. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell*. 2007;12:9-22.
18. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. *Nat Rev Drug Discov*. 2006;5:671-688.
19. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
20. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov*. 2009;8:627-644.
21. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer*. 2012;12:401-410.
22. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432:1032-1036.
23. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol*. 2009;22:124-131.
24. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol*. 2011;13:1016-1023.
25. Inoki K, Kim J, Guan KL. AMPK and mTOR in cellular energy homeostasis and drug targets. *Annu Rev Pharmacol Toxicol*. 2012;52:381-400.
26. Pattingre S, Levine B. Bcl-2 inhibition of autophagy: a new route to cancer? *Cancer Res*. 2006;66:2885-2888.
27. Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. *Genes Dev*. 2006;20:267-275.
28. Matoba S, Kang JG, Patino WD, et al. p53 regulates mitochondrial respiration. *Science*. 2006;312:1650-1653.
29. Sparks CA, Guertin DA. Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. *Oncogene*. 2010;29:3733-3744.
30. Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. *Cell Cycle*. 2011;10:2305-2316.
31. Guertin DA, Stevens DM, Saitoh M, et al. mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. *Cancer Cell*. 2009;15:148-159.
32. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol*. 2010;11:9-22.
33. Colombani J, Raisin S, Pantalacci S, Radimerski T, Montagne J, Léopold P. A nutrient sensor mechanism controls *Drosophila* growth. *Cell*. 2003;114:739-749.
34. Sarbassov D, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol*. 2005;17:596-603.
35. Bohn G, Allroth A, Brandes G, et al. A novel human primary immunodeficiency syndrome caused by deficiency of the endosomal adaptor protein p14. *Nat Med*. 2007;13:38-45.
36. Guertin DA, Sabatini DM. The pharmacology of mTOR inhibition. *Sci Signal*. 2009;2:pe24.
37. Thoreen CC, Kang SA, Chang JW, et al. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem*. 2009;284:8023-8032.
38. Sarbassov D, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell*. 2006;22:159-168.
39. Guertin DA, Sabatini DM. An expanding role for mTOR in cancer. *Trends Mol Med*. 2005;11:353-361.
40. Astrinidis A, Henske EP. Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease. *Oncogene*. 2005;24:7475-7481.
41. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell*. 2006;124:471-484.
42. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 2006;441:424-430.

Lydia W. S. Finley and Craig B. Thompson

13

The Metabolism of Cell Growth and Proliferation

Why Is Metabolism Important to an Understanding of Cancer?

At its heart, cancer is a disease of abnormal proliferation. Proliferation represents a distinct metabolic challenge: cells must replicate all of their proteins, lipids, and nucleic acids to generate a daughter cell. This process requires vast inputs of energy and raw materials. As a result, proliferating cells take up large quantities of nutrients in order to engage the biosynthetic reactions that support cell growth. Unsurprisingly, cancer cells exhibit many of the metabolic features that are characteristic of proliferating cells.[1] Given the logical association between metabolism and proliferation, it is not surprising that the study of cancer metabolism could yield diagnostic and therapeutic opportunities. Indeed, the propensity of cancer cells to take up high levels of glucose has been exploited clinically. For example, uptake of radioactive glucose analogs, measured by positron emission tomography, can be used to diagnose and monitor glucose-avid tumors.

Over the past decade, the study of cancer has become increasingly entwined with the study of cellular metabolism. Altered metabolism is a common feature of cancer cells, and most oncogene and tumor suppressor pathways directly regulate cellular metabolic pathways.[2] Moreover, cancer-associated metabolic alterations can in turn signal to alter the expression or activity of oncogenic pathways.[3] This chapter provides an overview of the metabolic changes associated with cancer cells, the genetic mechanisms that regulate tumor metabolism, and the clinical implications of altered cancer metabolism.

The Metabolic Requirements of Cell Proliferation

Proliferating and nonproliferating cells have very different metabolic requirements. Nonproliferating cells must maintain an ample supply of energy to fuel basic cellular processes, such as maintaining ion gradients and supporting transcription and translation, in addition to fulfilling their tissue-specific biological roles. This energy, usually in the form of adenosine triphosphate (ATP), is generated primarily in the mitochondrion. The basic building blocks of carbohydrates, lipids, and proteins (glucose, fatty acids, and amino acids, respectively) can be broken down in the tricarboxylic acid (TCA) cycle and the NADH generated by their oxidation used to produce ATP using oxidative phosphorylation (OXPHOS) (Figure 13-1).[4] This efficient conversion of nutrients into energy permits relatively low levels of nutrients to support the metabolic demands of quiescent cells.

In contrast, proliferating cells face the challenge of doubling all of the macromolecules in the cell in order to divide into two daughter cells. Cells must produce abundant nucleic acids, amino acids, and fatty acids in order to synthesize the DNA, proteins, and membranes that are necessary to replicate themselves. The process of synthesizing macromolecules, known as *anabolism* or *anabolic metabolism*, requires three main inputs that are critical for the process of cell growth:

1. *Substrates.* Extensive intracellular metabolic networks ensure that mammalian cells can generate many of the metabolic building blocks required for growth from relatively few inputs. For example, humans can synthesize 11 amino acids (which are consequently known as the nonessential amino acids) by rearranging the nitrogen and carbon backbones of other dietary inputs. (The other 9 amino acids are considered "essential" amino acids, as they cannot be synthesized in human cells.) In most cell types, glucose is the most important substrate for anabolic metabolism, because by-products of glucose breakdown (or catabolism) can contribute to the production of nonessential amino acids as well as nucleic acids and lipids. Most reduced nitrogen utilized for cell growth is taken into cells as glutamine. Glutamine plays a critical role providing nitrogen for nucleotide and nonessential amino

acid synthesis.[5] Like glucose, glutamine can also provide carbon units for fatty acid synthesis. Given their importance as central metabolites bridging both anabolic and catabolic pathways, glucose and glutamine are major fuels for cell proliferation and are discussed in detail later in this chapter. Cell proliferation requires numerous other substrates, including essential amino acids and a variety of vitamins and minerals, which will not be discussed (for more reading, see Ref. 6).

2. *Chemical energy*. Intramolecular bonds store large amounts of energy. Consequently, catabolism of macromolecules releases energy, which is harnessed either directly by driving the production of ATP or indirectly by reducing the electron carrier NAD^+ to NADH, which in turn fuels ATP production via OXPHOS. Conversely, macromolecular synthesis requires extensive energy input. Separately, protein translation and DNA replication consume significant amounts of ATP. In contrast to nonproliferating cells, a significant portion of the ATP required to sustain cell proliferation is produced by glycolysis, which is upregulated in rapidly dividing cells.

3. *Reducing equivalents*. Just as many catabolic reactions involve the oxidation, or removal of electrons from metabolites to electron carriers such as $NADP^+$ or NAD^+, several anabolic reactions require the input of electrons as reducing agents to forge intramolecular bonds. Reducing equivalents, primarily in the form of NADPH, carry these electrons for use in anabolic pathways. Fatty acid, nucleic acid, and nonessential amino acid synthesis consume large amounts of NADPH. The relative levels of $NADPH/NADP^+$ and $NADH/NAD^+$ often reflect the "redox" status of the cell—the extent to which the cell has more reduced electron carriers (higher $NAD(P)H/NAD(P)^+$ ratio) or more oxidized electron carriers (lower $NAD(P)H/NAD(P)^+$ ratio). Cellular redox balance is tightly controlled by many factors and can contribute to cancer growth, proliferation, and survival in numerous ways.[7] For proliferating cells, a large fraction of NADPH and NADH may be generated by glucose catabolism.[8]

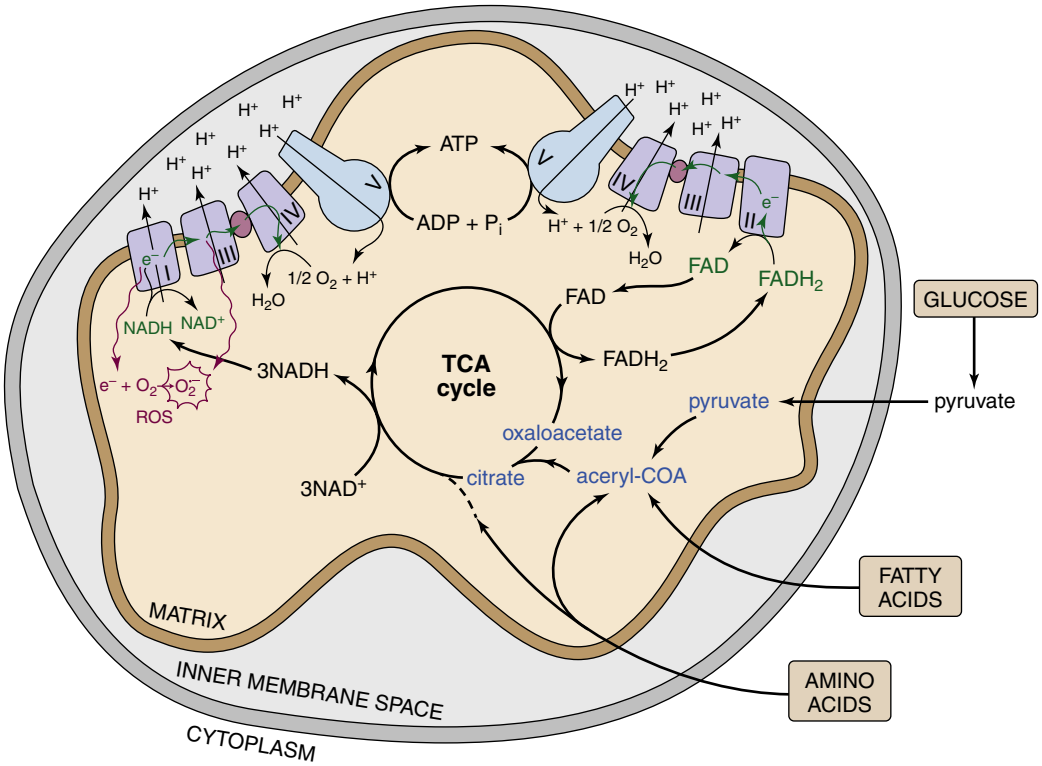


Figure 13-1 Mitochondria efficiently degrade metabolic fuels to generate ATP Metabolic fuels—carbohydrates, fats, and proteins—are broken down into their constituent units: glucose, fatty acids, and amino acids. These molecules are further degraded into metabolic intermediates that enter the tricarboxylic acid (TCA) cycle. Glucose-derived pyruvate, fatty acids, and many amino acids enter the TCA as acetyl-CoA, which condenses with oxaloacetate to form citrate. A series of biochemical reactions oxidize citrate back to oxaloacetate, while using the high-energy electrons released by these oxidation reactions to reduce the electron carriers NAD^+ and FAD to NADH and $FADH_2$, respectively. NADH and $FADH_2$ carry these electrons to complex I and complex II of the electron transport chain, regenerating NAD^+ and FAD. The high-energy electrons are passed through the electron transport chain (complex I-III-IV or complex II-III-IV), and their energy is used to pump protons from the mitochondrial matrix into the inner membrane space, generating an electrochemical gradient known as the *mitochondrial membrane potential*. Ultimately, the electrons reduce molecular oxygen to form water. The energy released by protons flowing down their electrochemical gradient through complex V/ATP synthase is harnessed to synthesize ATP from ADP and P_i. Reactive oxygen species (ROS) are formed when electrons leak out of the electron transport chain prematurely, combining with oxygen to form the superoxide radical ($O_2^{\cdot-}$). Although ROS is an inevitable by-product of oxidative phosphorylation, its production can be increased whenever flow through the electron transport chain is slowed, whether by heightened membrane potential or reduced oxygen concentrations. Metabolites are shown in *blue*. Electron carriers are shown in *green*.

Regulation of Cell Metabolism

During conditions of nutrient scarcity, anabolic programs are suppressed in favor of energy-generating catabolic pathways. Conversely, multiple cellular metabolic pathways must be coordinately rewired to support cell proliferation. Elevated nutrient uptake—particularly of glucose and glutamine—provides the substrates for cell growth. Rapid catabolism of glucose via glycolysis produces sufficient ATP and NADPH to support energy-dependent anabolic reactions while generating the metabolic intermediates that will be critical for macromolecular biosynthesis. Similarly, catabolism of glutamine will help maintain bioenergetics while ensuring adequate substrates for cell growth. Multiple regulatory mechanisms ensure that energy production and macromolecular biosynthesis are appropriately balanced with the metabolic needs of the cell.

Unicellular organisms are directly exposed to the environment and any fluctuations in nutrient availability that may occur. Consequently, these organisms have elegant mechanisms to sense the available nutrients and rewire their metabolism accordingly. Thus, for unicellular organisms, nutrient availability directly controls the signals that regulate growth and proliferation. If a unicellular organism is in an environment with abundant nutrients, these nutrients will directly activate signaling pathways that instruct the cell to engage anabolic metabolic pathways and to undergo cell division. Conversely, conditions of low nutrient availability will halt cell growth and division. Cells will engage catabolic pathways in order to produce energy to survive through the period of scarcity (Figure 13-2).

In contrast, metazoans have complex organ systems that maintain a relatively constant level of extracellular nutrients throughout the body. Cell-autonomous metabolic regulation would be catastrophic, as well-fed cells might undergo aberrant growth and proliferation. Thus, nutrient availability alone cannot determine whether cells engage anabolic or catabolic pathways; the metabolism of individual cells must be aligned with the needs of the organism as a whole. This coordination is largely achieved through extracellular growth factors that regulate nutrient uptake and utilization. Binding to receptors on the cell surface, growth factors stimulate intracellular signal-transduction cascades that regulate many facets of nutrient uptake and metabolism. In particular, growth factor signaling enables cells to take up high levels of nutrients such as glucose and glutamine and to engage in anabolic pathways supporting cell growth (see Figure 13-2).[9] In this manner, systemic signals can target individual cells (expressing the proper growth factor receptor) to induce specific activities, thereby ensuring that the behavior of individual cells is tailored to the needs of the entire organism. In the absence of growth factor signaling, metazoan cells are largely quiescent, maintaining homeostasis by the efficient degradation of the limited nutrients they are directed to take up. By engaging oxidative, catabolic pathways that preserve intracellular energy levels, these cells are able to survive and fulfill their allotted functions.

Most cells have the capacity to engage both anabolic and catabolic pathways, and the balance between the two—the balance that guards against inappropriate cell proliferation while maintaining cell survival—is carefully regulated by a number of factors. External signals such as growth factors instruct the cell to grow, activating anabolic reactions

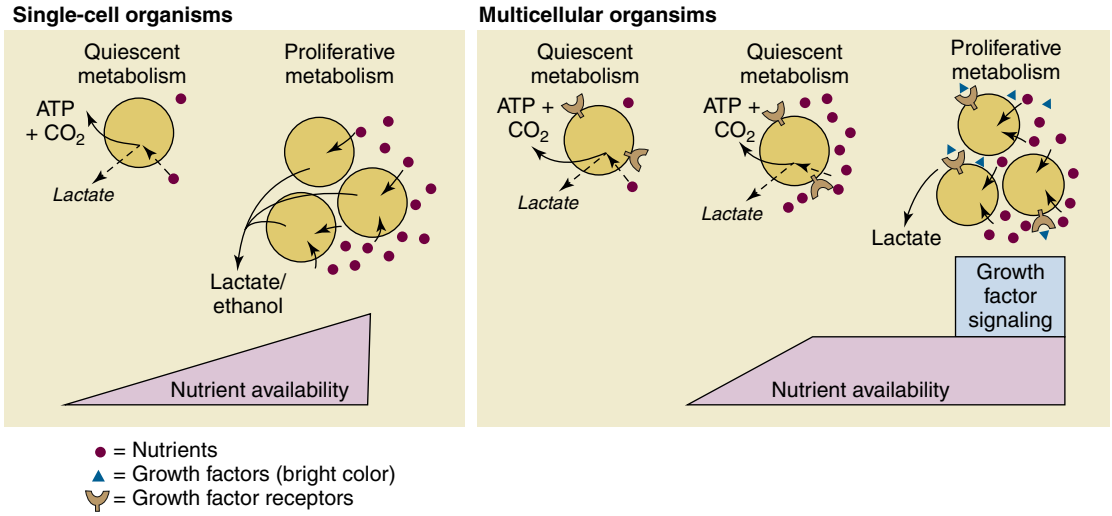


Figure 13-2 Proliferative metabolism is differentially regulated in single-cellular and multicellular organisms Quiescent cells in both single-cellular organisms and multicellular organisms engage in oxidative metabolism, efficiently converting available nutrients to ATP and carbon dioxide. In the presence of abundant nutrients, single-cellular organisms will engage in proliferative metabolism, consuming high levels of nutrients to produce biomass while excreting large amounts of lactate or other anaerobic fermentation products, such as ethanol. In contrast, the presence of abundant nutrients is insufficient to trigger proliferative metabolism in multicellular organisms, which require additional growth factor signals in order to engage proliferative metabolism. Once stimulated, these cells will undergo high rates of glycolysis to fuel cell growth.

and driving nutrient uptake accordingly. The activities of key metabolic enzymes that determine whether metabolites enter anabolic or catabolic pathways are regulated by post-translational modifications, most commonly phosphorylation triggered by growth factor signaling. Metabolites themselves function as critical allosteric regulators of metabolic enzyme activity, increasing or inhibiting flux through metabolic pathways according to the needs of the cell. For example, signals of abundant energy stores such as ATP and NADH can allosterically inhibit multiple enzymes that otherwise promote the channeling of metabolites into catabolic, energy-producing pathways. This allows for the diversion of metabolites toward anabolic processes when energy supply is plentiful. Similarly, both metabolic cues and growth factor signaling pathways can regulate the expression levels of metabolic enzymes. Through these mechanisms, cells carefully control the activation of anabolic or catabolic metabolic programs and ensure that during cell proliferation, metabolic pathways are coordinately rewired to support cell growth.

A major hallmark of cancer is the development of cell-autonomous regulation of cell growth.[10] Through multiple genetic events, including activation of growth factor signal transduction pathways (oncogenes) and loss of inhibitory signals (tumor suppressors), cancer cells circumvent dependence on external growth factor signaling. Constitutive activation of growth factor signaling pathways ensures that cancer cells are not subject to the normal regulation of metazoan cells. Consequently, cancer cells exhibit metabolic transformation, taking up high levels of nutrients and engaging in proliferative metabolism to support unchecked cell growth.

The Metabolic Profile of Cancer Cells

It is increasingly clear that cancer cells exhibit metabolic phenotypes that are similar to rapidly proliferating normal cells, with the major difference that the metabolic alterations in cancer cells stem from oncogenic cell-autonomous signaling, rather than the appropriate result of specific growth signals originating outside the cell. The characteristic metabolic features of rapidly proliferating tumor cells include elevated glucose uptake and glycolysis, increased glutamine uptake and utilization, and enhanced lipid and nucleotide biosynthesis. This section discusses the common metabolic signatures of tumor cells and how these metabolic alterations may support tumor growth.

Aerobic Glycolysis: The Warburg Effect

The most well-known and prevalent metabolic change associated with cancer cells is the enhanced uptake and metabolism of glucose, often referred to as the *Warburg effect*. In 1926, Otto Warburg noted that rapidly proliferating ascites cancer cells take up high levels of glucose and produce large amounts of lactate, even in the presence of oxygen. This finding was not intuitive: Work begun by Louis Pasteur had shown a clear inverse relationship between oxygen availability and the rate of glucose fermentation to lactate. The ability of eukaryotic cells to switch between anaerobic energy production through fermentation to aerobic energy production through oxidative phosphorylation depending on the presence of oxygen is known as the *Pasteur effect* (Figure 13-3). In cancer cells in the presence of oxygen, one might expect glucose to be metabolized to pyruvate, which would then be completely oxidized in the mitochondrion to produce ATP through the oxygen-dependent process of oxidative phosphorylation (see Figure 13-1). The surprising observation that cancer cells converted pyruvate to lactate despite abundant oxygen availability—the process of aerobic glycolysis—led Warburg to speculate that mitochondrial function is impaired in tumor cells, forcing a reliance on glycolytic metabolism.[11]

We now know that most cancers do not exhibit impaired mitochondrial energy production.[12] Moreover, research increasingly suggests that mitochondrial metabolic pathways are not simply catabolic and energy-producing; they may also play a critical role supporting anabolic biosynthetic pathways, as discussed later. These findings indicate that high aerobic glycolysis is not the secondary result of a metabolic failure, but rather a specific adaptation that promotes cell growth. Despite the centrality of the Warburg effect to cancer cell metabolism, there is still some debate as

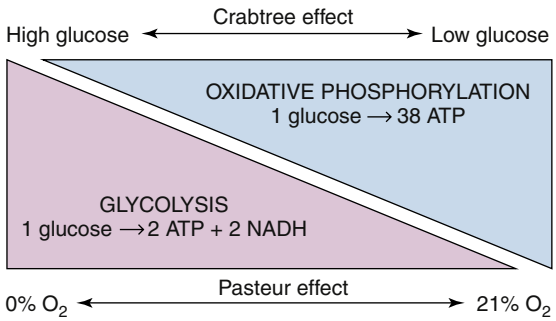


Figure 13-3 Normal, nonproliferating cells coordinate the balance between glycolysis and oxidative phosphorylation based on oxygen and glucose availability The Crabtree effect describes the inverse relationship between glucose availability and oxidative phosphorylation. In normal cells, abundant glucose promotes glycolytic metabolism and inhibits oxidative metabolism; conversely, low glucose availability stimulates mitochondrial oxidative phosphorylation to maximize ATP production from the available glucose. Similarly, the Pasteur effect summarizes the influence of oxygen tension on cellular metabolism: as cells adapt to low oxygen, they reduce their reliance on oxygen-dependent oxidative phosphorylation and increase their glycolytic rate to compensate. The Warburg effect appears to contradict these relationships, as highly proliferative cells undergo glycolytic metabolism even in the presence of abundant oxygen.

to how aerobic glycolysis confers a proliferative advantage to tumor cells.

The Paradox of the Warburg Effect

Glycolysis comprises a series of reactions that convert 1 molecule of glucose to 2 molecules of pyruvate, generating 2 molecules each of ATP and NADH (Figure 13-4). If oxygen is present, pyruvate is converted to acetyl-CoA in the mitochondrion, and acetyl-CoA is oxidized by the TCA cycle, producing 1 molecule of GTP and four pairs of high-energy electrons that will be used to fuel OXPHOS (3 molecules of NADH and 1 molecule of $FADH_2$). As the glycolytic reactions occur in the cytosol, the reducing equivalents of the NADH generated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) must be transferred to the mitochondrion in order for their energy to be harnessed into ATP. The malate-aspartate shuttle and the glycerol-3-phosphate shuttle transfer the electrons into the mitochondrion for oxidation by the electron transport chain. All together, this process of complete glucose oxidation produces 38 molecules of ATP. Thus, when oxygen is present, glucose can be efficiently converted to ATP while regenerating the NAD^+ required to maintain glycolysis.

If NADH is not oxidized to NAD^+, the subsequent depletion of NAD^+ will inhibit GAPDH and block glycolysis. To avoid this, under anaerobic conditions lactate dehydrogenase (LDH) reduces pyruvate to lactate, consuming NADH and producing NAD^+. In total, therefore, anaerobic glycolysis yields two molecules of ATP and two molecules of lactate that are secreted from the cell.

The paradox of the Warburg effect is that cells convert pyruvate to lactate even in the presence of oxygen. If glucose oxidation produces 19 times more ATP than anaerobic glycolysis, what benefit could a cell gain from choosing the less efficient route? And why would a cell that is growing rapidly throw away valuable carbon units in the form of lactate? Warburg's studies described an astonishing rate of lactate production in ascites tumor cells—cells produced up to

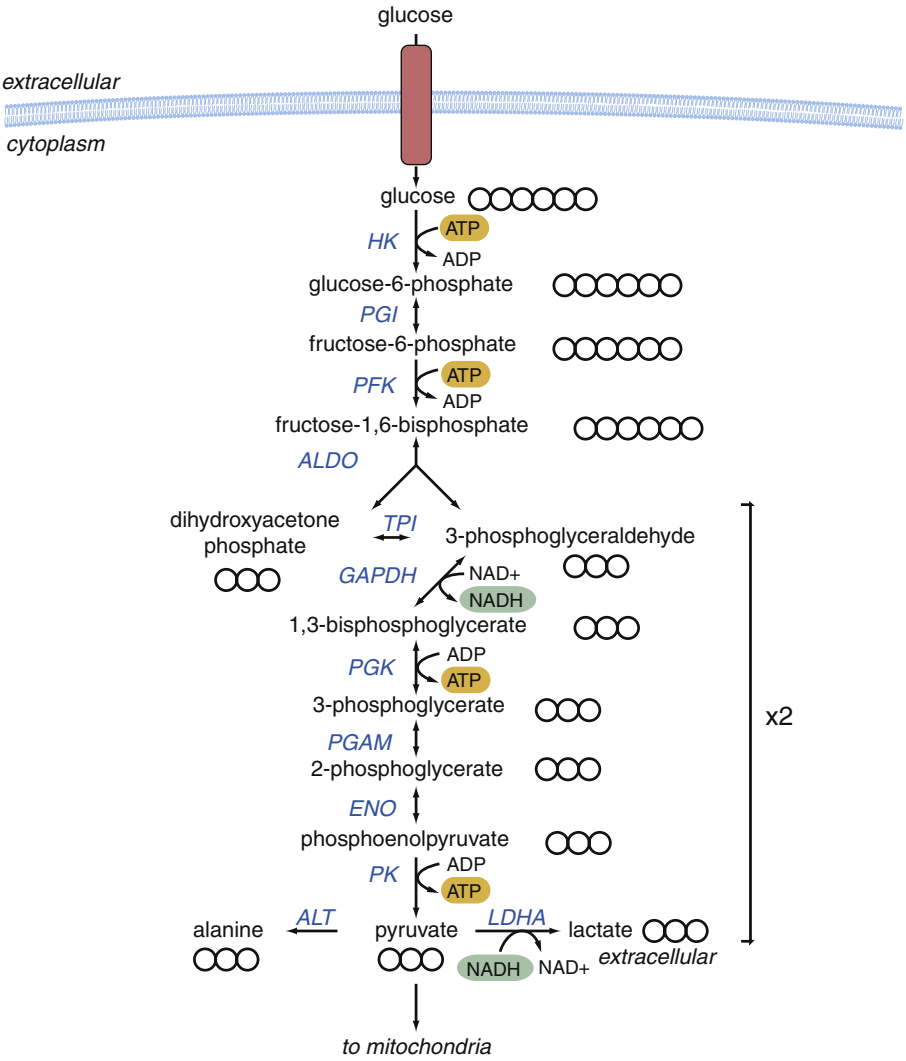


Figure 13-4 Glycolysis produces 2 ATP and 2 NADH for each molecule of glucose Glucose is metabolized to pyruvate through a series of biochemical reactions in the cytosol of the cell. This process yields a net of 2 ATP and 2 NADH molecules for each molecule of glucose. Pyruvate can be converted to lactate to regenerate the NAD^+ required to maintain flux through glycolysis, transaminated to alanine, or transported to the mitochondrion for further oxidation. Circles represent the number of carbons in each metabolite. Enzymes are shown in *blue;* reduced NADH is shown in *green;* ATP is shown in *orange*. *ALDO,* aldolase; *ALT,* alanine transaminase; *ENO,* enolase; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *HK,* hexokinase; *LDHA,* lactate dehydrogenase; *PFK,* phosphofructokinase; *PGAM,* phosphoglycerate mutase; *PGI,* phosphoglucose isomerase; *PGK,* phosphoglycerate kinase; *PK,* pyruvate kinase; *TPI,* triosephosphate isomerase.

30% of their dry weight in lactic acid per hour.[13] Although it is now known that this is a much higher rate than for most tumors, it is clear that aerobic glycolysis occurs at a high rate in many tumor cells. There are several potential explanations for how tumors could sustain such apparently wasteful metabolism and the benefits that rapid aerobic glycolysis might bestow:

1. *Aerobic glycolysis is a source of rapid ATP generation.* According to Warburg's calculations, the high rate of glucose consumption enabled cells to produce approximately the same amount of ATP through fermentation as through respiration.[11] Similarly, a series of studies demonstrated that stimulating cells to proliferate increased ATP turnover, as expected; however, the increased ATP demand was met entirely by increased glycolytic flux and not by any increase in ATP production by oxidative phosphorylation.[14] Thus, the relative inefficiency of ATP production may be counterbalanced by the high rate of glucose consumption. Furthermore, tumor cells may extract more than two molecules of ATP per molecule of glucose: Several studies demonstrate that cytosolic-mitochondrial NADH shuttles are active in tumor cells, indicating that tumors can oxidize GAPDH-derived NADH in the mitochondrion to produce ATP.[15-18] In addition, glycolysis generates cytosolic ATP very rapidly: The conversion of glucose to lactate can take mere seconds. This could benefit tumor cells undergoing high rates of protein, lipid, and nucleotide biosynthesis. Thus, as long as glucose supplies are not limited, high levels of glycolysis may provide an advantageously rapid and plentiful source of ATP.
2. *Adaptation to hypoxia.* Cells in solid tumors experience notoriously harsh and varied metabolic conditions as tumor growth disrupts the normal tissue architecture. Blood vessels inside the tumors are often dysfunctional or nonexistent. Consequently, tumor cells commonly experience periods of intermittent hypoxia. Survival in hypoxia requires metabolic adaptations, including increasing glycolysis and downregulating mitochondrial fuel oxidation.[19] A cell already primed with these metabolic adaptations would be more likely to survive inside a solid tumor. Indeed, cells cultured ex vivo from solid tumors often display high lactate production even when well oxygenated, suggesting that elevated glycolysis is a fundamental feature that either predisposes cells toward tumor formation or is selected for early in tumor development. It is worth noting that because hypoxia typically results from inadequate blood supply, cells rarely experience hypoxia without concomitant deprivation of nutrients such as glucose that are also provided by the vasculature. Thus, although glycolytic metabolism may increase the likelihood of survival during periods of intermittent hypoxia, sustained hypoxia will likely negatively influence cell growth regardless of metabolic adaptations.
3. *Acidification of microenvironment.* There is some evidence that the apparently wasteful secretion of lactate may itself confer a selective advantage to tumor development. Cells export lactate through the family of H^+-linked monocarboxylate transporters. By acidifying the microenvironment, lactate export may promote the death of normal cells and extracellular matrix degradation to enhance tumor invasion.[20] In support of this model, studies suggest that acidic conditions can stimulate tumor cell invasion in vitro, and in vivo interventions to increase pH can reduce spontaneous metastases.[21] Intriguingly, some studies suggest that lactate may also be used as a fuel source. Lactate exported from hypoxic cells can be used as a substrate for oxidative metabolism in normoxic cells within the same tumor, preserving glucose for the hypoxic cells while providing fuel for the normoxic cells.[22] Thus, lactate production may not be an unfortunately costly by-product of glycolytic metabolism, but may also serve pro-tumorigenic roles. Indeed, blocking conversion of pyruvate to lactate by suppressing LDH expression can impair tumorigenesis.[23] Clearly, the production of lactate is critical for cancer cell growth, whether by maintaining oxidized NAD^+ to promote glycolysis, acidifying tumor surroundings to promote cancer cell survival, or providing a means for efficient substrate allocation in a metabolically diverse microenvironment.

Glycolysis Provides Key Intermediates to Support Cell Growth

The preceding examples provide clues for how rapid aerobic glycolysis might provide a growth or survival advantage to tumor cells. However, we now know that aerobic glycolysis as Warburg described it—the conversion of glucose to lactate—is not the exclusive fate of glucose in cancer cells. Glucose is a major source of many of the building blocks of macromolecular biosynthesis, and it is clear that these various anabolic fates of glucose are an important component of the elevated glycolysis in cancer cells.

Glucose-Derived Metabolites Contribute to All Classes of Macromolecules

Glycolytic flux provides substrates for biosynthetic reactions that are required for the synthesis of lipids, nucleotides, and proteins (Figure 13-5). Glycolytic intermediates can be diverted to the pentose phosphate pathway to produce ribose 5-phosphate, which serves as the foundation for the de novo synthesis of purines and pyrimidines. The oxidative arm of the pentose phosphate pathway also generates NADPH,

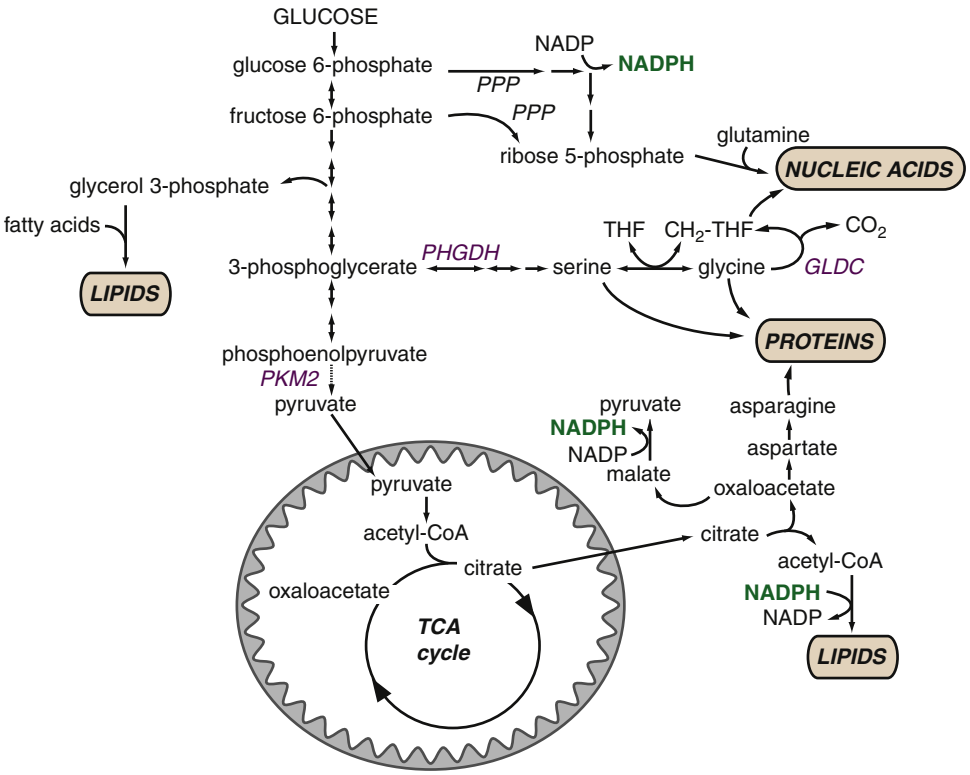


Figure 13-5 Glucose metabolism provides critical intermediates for cell growth Glycolytic intermediates provide the substrates for the pentose phosphate pathway (PPP) that generates the ribose 5-phosphate that is critical for nucleotide biosynthesis. The oxidative arm of the PPP, which utilizes glucose 6-phosphate, additionally generates NADPH. Glycerol 3-phosphate, which contributes the glycerol head groups for phospholipid biosynthesis, is formed from the intermediate dihydroxyacetone phosphate. 3-Phosphoglycerate provides the foundation for the serine synthesis pathway, which can further fuel glycine production for protein synthesis. This pathway also contributes to the pool of one-carbon units (CH_2-THF) that are used in nucleotide biosynthesis. Glucose-derived citrate provides the acetyl-CoA that represents the fundamental building block for the synthesis of the fatty acids that comprise cellular lipids. Key metabolic enzymes are shown in *blue;* NADPH required for biosynthetic reactions is shown in *green*. *CH_2-THF,* methylene tetrahydrofolate; *GLDC,* glycine decarboxylase; *PHGDH,* phosphoglycerate dehydrogenase; *PKM2,* pyruvate kinase M2 isoform; *PPP,* pentose phosphate pathway; *THF,* tetrahydrofolate.

which can provide reducing equivalents for nucleotide and fatty acid synthesis. Similarly, the glycerol required for phospholipid synthesis is derived from the glycolytic intermediate dihydroxyacetone phosphate. As the major component of mammalian cell membranes, phospholipid synthesis is critical for cell growth. Glycolytic intermediates can also contribute to amino acid synthesis: 3-Phosphoglycerate can be converted to serine and ultimately glycine.

A series of recent studies documenting tumors whose growth depends on diversion of glycolytic flux to anabolic pathways lends support to the hypothesis that glycolytic intermediates play a key role fueling tumor growth. Phosphoglycerate dehydrogenase (PHGDH), the enzyme that catalyzes the first step of the serine synthesis pathway that branches from glycolysis, is frequently amplified in melanoma and breast cancers, and flux into the serine synthesis pathway can support tumor cell growth.[24,25] Similarly, multiple tumor types display high levels of glycine decarboxylase (GLDC), which generates methylene tetrahydrofolate necessary for pyrimidine synthesis, and GLDC expression can promote cellular transformation and tumorigenesis.[26]

Macromolecular Synthesis, Not ATP, May Be the Major Purpose of Elevated Glycolysis in Cancer Cells

The importance of glycolytic intermediates as biosynthetic building blocks supporting cell proliferation was underscored by the observation that most proliferating cells express the M2 isoform of pyruvate kinase (PK), whereas quiescent cells express the PKM1 isoform.[27] Pyruvate kinase catalyzes the final ATP-producing step of glycolysis, converting phosphoenolpyruvate to pyruvate. Perhaps counterintuitively, PKM2 has less intrinsic activity than PKM1; PKM2 is also inhibited by growth factor signaling. These observations suggest that *inhibiting* the final step of glycolysis can be beneficial for cell growth. By slowing the final step of glycolysis, PKM2 enables accumulation of upstream glycolytic intermediates that can then be diverted into biosynthetic pathways. These findings indicate that proliferating cells do not undergo glycolysis for the sole purpose of ATP production; rather, they appear to selectively slow pyruvate production in order to maximize the flow of glucose carbons into anabolic pathways.

Expression of PKM2 may have the added benefit of reducing ATP production. Although ATP is required for anabolic reactions, NADPH is quantitatively more important. Biosynthesis of many macromolecules requires more reducing equivalents than energy from ATP.[1] Consequently, if all glucose were oxidized completely, ATP production would far outstrip NADPH production, thus greatly circumscribing potential cell growth. Furthermore, ATP is a potent inhibitor of phosphofructokinase-1, a key enzyme in the regulation of glycolysis, and so ATP produced in excess of biosynthetic demands would have the adverse effect of blocking glycolysis, further reducing NADPH production.

Although cancer cells preferentially reduce pyruvate kinase activity, the final step in glycolysis cannot be abrogated completely, as pyruvate fulfills many important metabolic roles. The pyruvate produced at this step has three fates: It can be transaminated to alanine, reduced to lactate to regenerate NAD^+, or imported to the mitochondrion, where it is converted to acetyl-CoA that can enter the TCA cycle. In many tumors, glucose consumption is high enough to support anabolic pathways while also maintaining pyruvate flux into the mitochondria and still resulting in high amounts of lactate secretion. It is likely that reducing pyruvate kinase activity slows glucose flux through glycolysis in order to increase the likelihood that glucose can be diverted into anabolic pathways rather than being completely and rapidly exported as lactate or oxidized in the mitochondria. Given the high rates of lactate produced from glucose (as much as 90% of glucose is converted to lactate and alanine in glioblastoma cells),[28] cancer cells still have appreciable pyruvate consumption. It is likely that despite the high percentage of pyruvate that is secreted as lactate, the rate of glycolysis is so high that sufficient pyruvate remains to fuel the TCA cycle.

The TCA Cycle as a Biosynthetic Hub

Just as glycolytic intermediates are harnessed to support anabolic pathways, proliferating cells rewire the TCA cycle to prioritize cell growth over ATP generation. In its traditional form, the TCA cycle begins with the condensation of pyruvate-derived acetyl-CoA with oxaloacetate to form citrate (Figure 13-6). A series of reactions in the mitochondrial matrix convert citrate back to oxaloacetate, releasing two carbons as two molecules of carbon dioxide and producing three

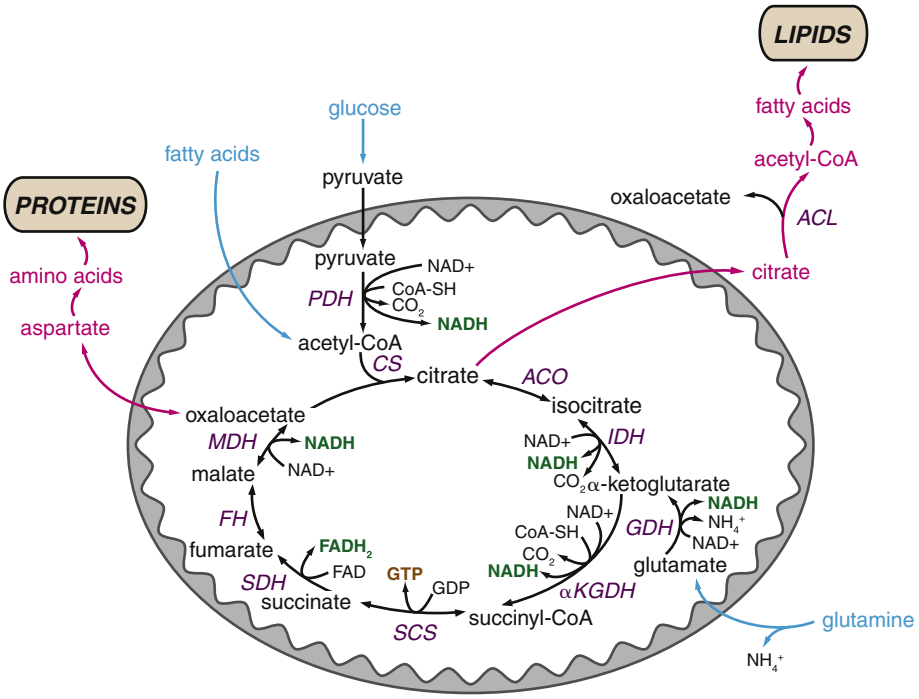


Figure 13-6 The tricarboxylic acid (TCA) cycle supports macromolecular biosynthesis Pyruvate derived from glucose enters the mitochondrion and is converted to acetyl-CoA by pyruvate dehydrogenase (PDH), and fatty acids are oxidized to produce acetyl-CoA units within the mitochondrion. Acetyl-CoA condenses with oxaloacetate to form citrate, which proceeds through the TCA cycle in highly oxidative tissues, releasing two carbons as carbon dioxide and regenerating oxaloacetate for further cycling. These reactions maximize ATP production from acetyl-CoA by reducing the electron carriers NADH and $FADH_2$ to fuel the electron transport chain. In proliferating cells, citrate is also exported from the mitochondrion to the cytosol, where it can provide the acetyl-CoA required for the synthesis of fatty acids and cholesterol. Similarly, oxaloacetate can form aspartate and other amino acids, contributing to intracellular amino acid pools for protein synthesis. Both of these reactions deplete TCA cycle intermediates; consequently, cells must replenish TCA cycle metabolites in order to regenerate the oxaloacetate necessary for citrate synthesis. Most cells use glutamine to fulfill this anaplerotic role: glutamine is converted to glutamate and then α-ketoglutarate, preserving TCA cycle flux. Metabolites that fuel the TCA cycle are shown in *light blue*. Metabolite efflux from the TCA cycle is shown in *pink*. Enzymes are shown in *dark blue*, reduced electron carriers in *green,* and high-energy triphosphates in *orange*. *PDH,* pyruvate dehydrogenase; *ACL,* ATP-citrate lyase; *ACO,* aconitase; *CoA-SH,* coenzyme A; *CS,* citrate synthase; *FH,* fumarate hydratase; *GDH,* glutamine dehydrogenase; *IDH,* isocitrate dehydrogenase; *αKGDH,* α-ketoglutarate dehydrogenase; *MDH,* malate dehydrogenase; *SCS,* succinyl-CoA synthetase; *SDH,* succinate dehydrogenase.

molecules of NADH and one molecule of $FADH_2$. NAD^+ and FAD are regenerated when NADH and $FADH_2$ donate their electrons to support oxidative phosphorylation. The cycle can continue by combining the recycled oxaloacetate with a new molecule of acetyl-CoA. In this manner, the oxidation of acetyl-CoA is used to maximize ATP production.

TCA Cycle Metabolites Are Required for Macromolecular Biosynthesis

In proliferating cells, citrate is not only oxidized in the TCA cycle but also can be exported to the cytosol where it is converted back to oxaloacetate and acetyl-CoA by the enzyme ATP citrate lyase (ACL). Cytosolic acetyl-CoA provides the substrate for the synthesis of fatty acids, cholesterol, and prostaglandins (Figure 13-7). Through this pathway, glucose acts as the major substrate for de novo lipogenesis; consequently, ACL inhibition blocks cell proliferation and inhibits tumor growth.[28-30]

Citrate is not the only TCA cycle metabolite that has an important biosynthetic role. Oxaloacetate and α-ketoglutarate can provide the carbon backbone for nonessential amino acids, which are used for protein and nucleic acid synthesis (see Figure 13-6). In this manner, multiple TCA cycle metabolites are diverted to other pathways that support cell growth.

Glutamine Plays Several Roles Supporting the TCA Cycle and Anabolic Metabolism

Although efflux of TCA cycle metabolites supports anabolic reactions, this results in depletion of TCA cycle intermediates from the mitochondrial matrix. Growing cells must draw on alternative sources to maintain the

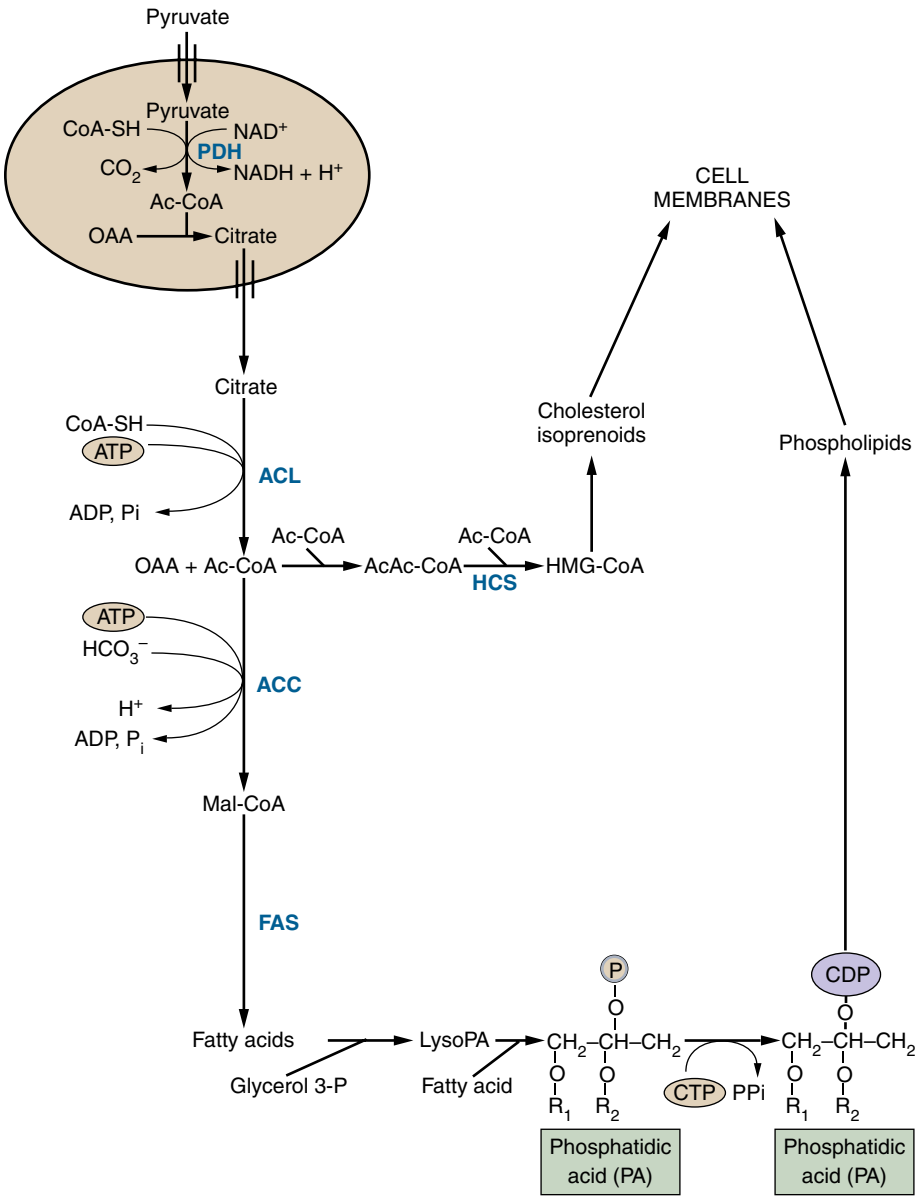


Figure 13-7 Glucose-derived acetyl-CoA fuels lipid and sterol synthesis Pyruvate derived from glycolysis can generate citrate in the mitochondrion. This citrate can be exported to the cytosol to provide acetyl-CoA for the synthesis of fatty acids, phospholipids, cholesterol, and isoprenoids, all of which are critical for membrane biogenesis and function. Metabolites are in *black;* enzymes are in *blue*. *ACC,* Acetyl-CoA carboxylase; *Ac-CoA,* acetyl-CoA; *ACL,* ATP-citrate lyase; *CDP,* cytidine diphosphate; *CoA-SH,* coenzyme A; *CTP,* cytidine triphosphate; *FAS,* fatty acid synthetase; *glycerol-3-P,* glycerol 3-phosphate; *HCS,* HMG-CoA synthetase; *HMG-CoA,* 3-hydroxy-3-methylglutaryl CoA; *lyso PA,* lysophosphatidic acid; *Mal-CoA,* malonyl-CoA; *OAA,* oxaloacetate; *Pi,* inorganic phosphate; *R,* acyl group on a lipid molecule.

oxaloacetate required for citrate synthesis. Replenishment of TCA cycle intermediates, a process known as *anaplerosis*, is thus critical to maintain both a constant supply of metabolites for synthesis and appropriate ATP production through oxidative phosphorylation. Most proliferating cancer cells meet their anaplerotic needs through the catabolism of glutamine (see Figure 13-6). Glutamine is converted to the TCA cycle intermediate α-ketoglutarate through a metabolic pathway known as glutaminolysis. Tumor cells take up high levels of glutamine, often in great excess of any other amino acid, and studies using carbon labeling techniques have demonstrated that glutamine is the major source of carbon for the cellular oxaloacetate pool in cancer cell lines.[28]

By maintaining flux through the TCA cycle—and therefore delivery of electrons to the electron transport chain—glutamine plays a critical role maintaining mitochondrial bioenergetics in many cancer cells. Consequently, many cancer cell lines are absolutely dependent on glutamine for survival. Addition of cell-permeable TCA cycle analogs can rescue death of glutamine-deprived cells, highlighting the importance of glutamine as an anaplerotic substrate in cancer cells.[31,32]

Proliferating cells rely on glutamine to fulfill additional biosynthetic roles. First, glutamine provides an important source of nitrogen for synthesis of nonessential amino acids and nucleotides. Glutamine is required for two independent steps in purine nucleotide synthesis, and oxaloacetate-derived aspartate is required for a third (Figure 13-8). Similarly, two

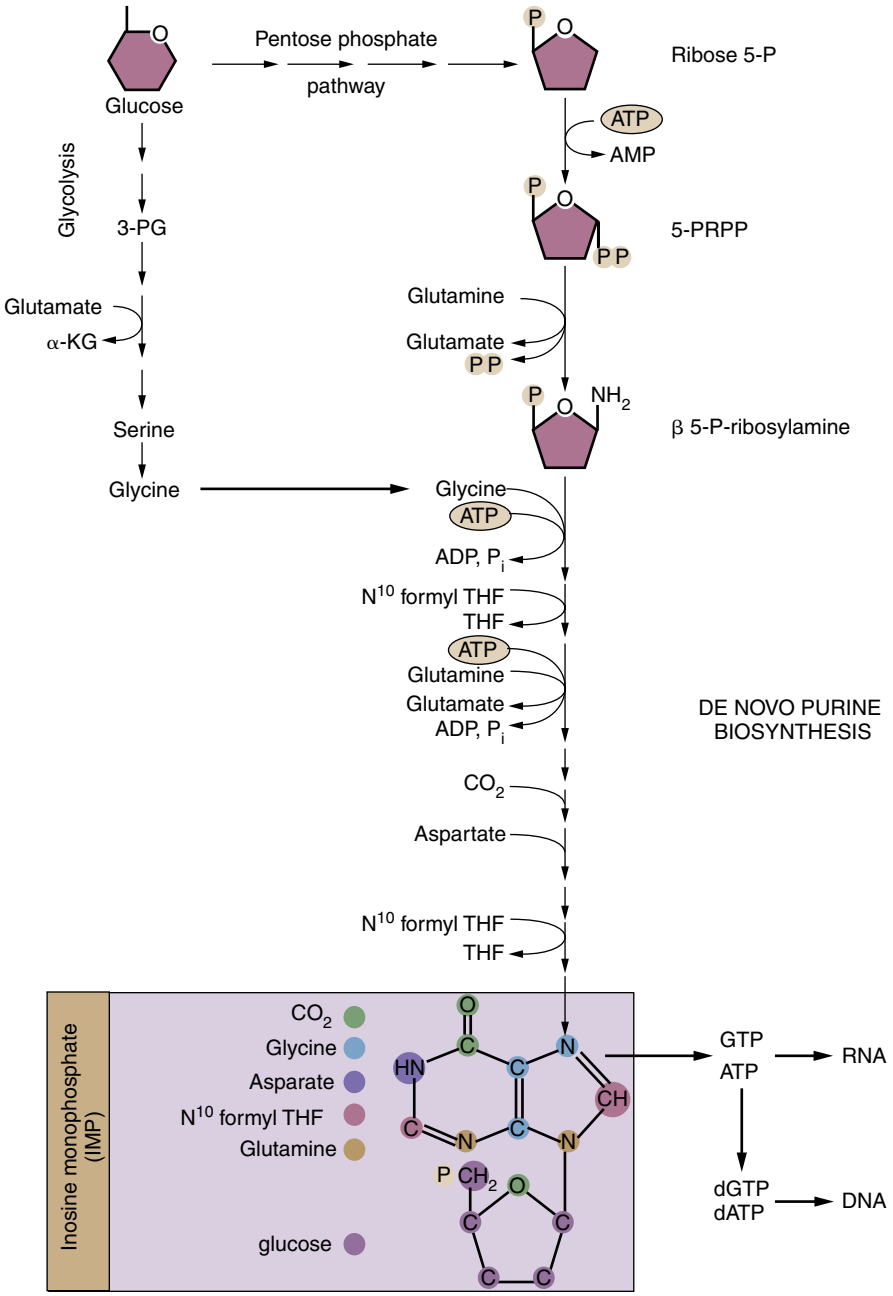


Figure 13-8 Nucleotide biosynthesis requires multiple metabolic inputs Nucleotide biosynthesis requires inputs from several metabolic pathways, highlighting why cells must coordinately regulate multiple metabolic pathways during proliferation. The de novo synthesis of purines (shown) and pyrimidines requires glucose, several amino acids, and one-carbon groups from folate metabolism. The origin of individual carbons and nitrogens on inosine monophosphate (IMP), the precursor to GTP and ATP, are color-coded in the purple box. *α-KG*, α-ketoglutarate; *N^{10} formyl THF*, N^{10} formyl tetrahydrofolate; *3-PG*, 3-phosphoglycerate; *β 5-P-ribosylamine*, β 5-phosphate-ribosylamine; *PRPP*, 5-phosphoribosyl pyrophosphate; *ribose 5-P*, ribose 5-phosphate; *P*, phosphate group.

steps of pyrimidine synthesis require glutamine. In all cases, glutamine donates nitrogen in the form of an amide group and is converted to glutamic acid, which provides a major source of nitrogen for amino acid synthesis. Transaminases can transfer the amine group from glutamic acid to α-ketoacids, which are themselves derived from the catabolism of glucose or glutamine, producing alanine, serine, aspartate, and ornithine. In turn, these amino acids act as precursors for the synthesis of glycine, cysteine, arginine, and asparagine. Likewise, two enzymatic reactions directly convert glutamate to proline. In this manner, glucose and glutamine contribute to the synthesis of every nonessential amino acid except for tyrosine, which is directly produced from the essential amino acid phenylalanine.

Intriguingly, cancer cells may convert up to 60% of glutamine carbon into lactic acid, an ostensibly wasteful secretion analogous to the Warburg effect.[28] One possible explanation for this behavior is that the conversion of glutamine to lactate produces NADPH, which is absolutely required for many anabolic reactions. NADPH is produced when malic enzyme converts glutamine-derived malate to pyruvate, which is subsequently reduced to lactate and secreted. In this manner, glutamine may contribute to all three needs of proliferating cells: Glutamine can provide the carbon and nitrogen for most metabolic building blocks, maintain ATP production to support bioenergetics, and generate reducing equivalents required for many anabolic reactions.

TCA Cycle Rearrangements Highlight the Role of TCA Cycle Metabolites as Biosynthetic Precursors

In proliferating cancer cells, the TCA cycle can behave more as a source of anabolic substrates rather than a bona fide cycle. A prime example of how the TCA cycle prioritizes biosynthetic reactions is the reductive carboxylation of α-ketoglutarate to isocitrate. During conditions of hypoxia or mitochondrial dysfunction, NADH accumulates from a combination of increased glycolysis and reduced oxidation of NADH by the electron transport chain. Depletion of oxidized NAD^+ poses a bioenergetic challenge for the cell: Both the conversion of glucose-derived pyruvate to acetyl-CoA and the production of oxaloacetate through the enzymes of the TCA cycle require NAD^+. Furthermore, during periods of acute hypoxia, pyruvate is converted to lactate at the expense of acetyl-CoA. Both of these events would strongly impair citrate production and thus cell growth. How, then, do hypoxic cells proliferate?

Several groups have demonstrated that under these conditions, the TCA cycle can partially function in reverse (for examples, see Refs. 33-35). Normally, isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, consuming NAD^+ and producing NADH. When NAD^+ is limiting, both the mitochondrial and cytosolic isoforms of IDH can function in reverse, carboxylating α-ketoglutarate to isocitrate and producing oxidized $NADP^+$ (Figure 13-9). In this manner, cells can use glutamine-derived α-ketoglutarate to generate citrate, providing a mechanism to maintain anabolic reactions even under hypoxic conditions that characterize the tumor microenvironment.

Genetic Mechanisms Driving Cancer Cell Metabolism

Metabolic transformation is a common feature of tumorigenesis, occurring in many tumor types in diverse tissues. It is logical

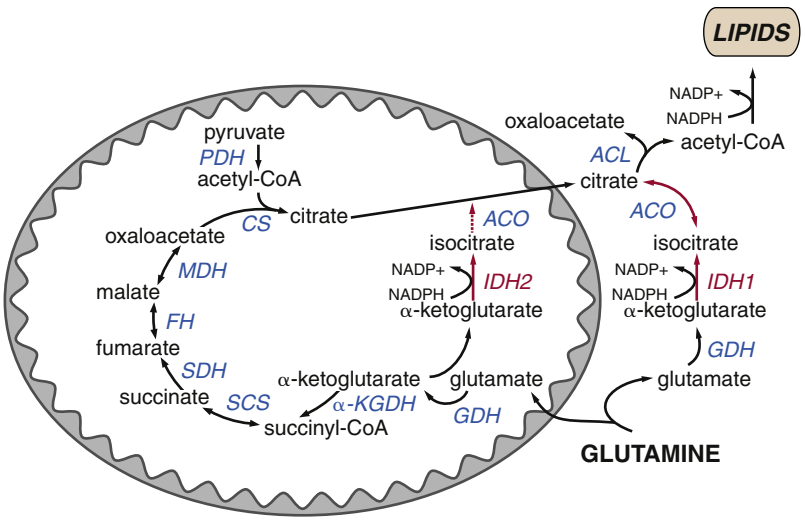


Figure 13-9 Reductive carboxylation supports lipid synthesis during hypoxia Citrate levels can be maintained even in cells with defective TCA cycles or during periods of hypoxia by the reductive metabolism of glutamine-derived α-ketoglutarate to citrate. Both mitochondrial and cytosolic isoforms of isocitrate dehydrogenase (IDH2 and IDH1, respectively) can function in reverse, reducing α-ketoglutarate to isocitrate and oxidizing NADPH to NADP+. Isocitrate is reversibly converted to citrate, which can be used to generate acetyl-CoA for lipid synthesis in the cytosol.

that metabolic transformation and oncogenic transformation would go hand in hand: As cancer cells acquire mutations that enable pathological proliferation, they must also acquire the means to support cell growth. Thus, many of the mutations that promote cancer growth also trigger metabolic reprogramming. This section discusses several common genetic oncogenic events that reprogram metabolism to support tumor growth.

Activation of the PI3K Pathway Promotes Metabolic Transformation

In normal cells, extracellular cues regulate the intracellular signaling pathways that tightly coordinate metabolism and proliferation. In many cells, this coordination is accomplished by the phosphatidylinositol-3-kinase (PI3K) pathway, a highly conserved signaling pathway that responds to a variety of extracellular cues. When growth factors bind to their cell surface receptors, receptor tyrosine kinases (RTKs) activate PI3K, which phosphorylates phosphatidylinositol lipids at the plasma membrane. These phosphorylated lipids recruit and activate downstream effectors of the PI3K pathway, triggering an intracellular signaling cascade that promotes cell growth, survival, and metabolism.

Constitutive activation of PI3K provides a growth stimulus and is therefore a major mechanism of tumorigenesis. In normal cells, PI3K activity is tightly guarded by a number of factors, ensuring that pathway activity is set at appropriate levels. RTK activity is strictly controlled by growth factor availability. In addition, negative feedback loops prevent prolonged pathway activation: The lipid phosphatase PTEN dephosphorylates phosphatidylinositol species to dampen PI3K signaling.

Cancers exhibit a variety of mutations to circumvent this regulation and gain cell-autonomous activation of the PI3K pathway.[36] Amplifications or mutations of growth factor receptors occur in a variety of cancers. Common examples are the amplification of the Her2/Neu receptor in breast cancer and epithelial growth factor receptor (EGFR) in lung cancer. Similarly, deletion, downregulation, or loss of function of PTEN increases cancer susceptibility and promotes tumor progression. Several tumor types display activating mutations in the catalytic PI3K subunit, PIK3CA; likewise, amplification of PIK3CA and the major PI3K effector, Akt, are common oncogenic events. All together, genetic activation of the PI3K pathway is one of the most prevalent classes of tumorigenic mutations.

Whereas PI3K signaling influences numerous cellular functions, the serine/threonine kinase Akt and its effector, mechanistic target of rapamycin (mTOR), are the PI3K effectors most commonly implicated in tumorigenesis. Akt coordinates cell growth and survival in a large part by rewiring metabolism to promote nutrient uptake and biosynthetic activities. Certainly, with regard to the regulation of cell metabolism, Akt activation is likely the most important consequence of PI3K pathway activation.

Akt induces many phenotypes consistent with the Warburg effect. Akt promotes the expression and membrane localization of glucose transporters, driving increased glucose uptake. Akt concurrently increases the expression of hexokinase, the enzyme that phosphorylates glucose so that it is retained in the cell and can undergo glycolysis. Akt regulates multiple steps of glycolysis through both gene expression and modulation of enzyme activity. Together, these activities result in a large increase in glycolytic flux in cells with active Akt. Similarly, both Akt and mTOR can trigger expression of lipogenic genes and lipid synthesis.[7,37] mTOR additionally promotes biosynthetic pathways by directly stimulating mRNA translation and ribosome biogenesis.[38] Thus, PI3K signaling is often sufficient to enhance the metabolic activities that support cell growth.

LKB1 and AMPK Are Metabolic Brakes That Circumvent Tumor Growth

While growth factor–stimulated signaling cascades promote cell growth under favorable conditions, cells have sophisticated nutrient sensing systems that serve to block growth when the internal energy supply is limiting. These regulators ensure that, during periods of intracellular nutrient depletion, metabolites are redirected from anabolic pathways and instead used to fuel catabolic pathways that will provide the energy required to survive the period of nutrient limitation. The AMP-activated protein kinase (AMPK) plays a major role coordinating cellular energy status with appropriate metabolic responses.

AMPK directly senses cellular energy levels in the form of the AMP/ATP ratio (Figure 13-10). Falling energy levels increase the cellular AMP/ATP ratio, priming AMPK for activation by the liver kinase B1 (LKB1). AMPK phosphorylates multiple targets with the cumulative effect of blocking anabolic reactions and stimulating energy-generating catabolic pathways. For example, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), with the dual effect of blocking fatty acid synthesis and activating fatty acid oxidation. AMPK also directly inhibits cell growth, both by inducing a p53-dependent cell cycle arrest and by blocking mTOR activity at multiple levels (see Figure 13-10). Through these diverse activities, AMPK functions as a metabolic checkpoint, ensuring that cell growth is halted until bioenergetic conditions are favorable for growth.

This is particularly relevant in the tumor microenvironment, when varied nutrient delivery could occasionally activate such metabolic checkpoints and block growth. Suggestively, LKB1 is classified as a tumor suppressor. Inactivating mutations in LKB1 cause Peutz-Jeghers syndrome, a

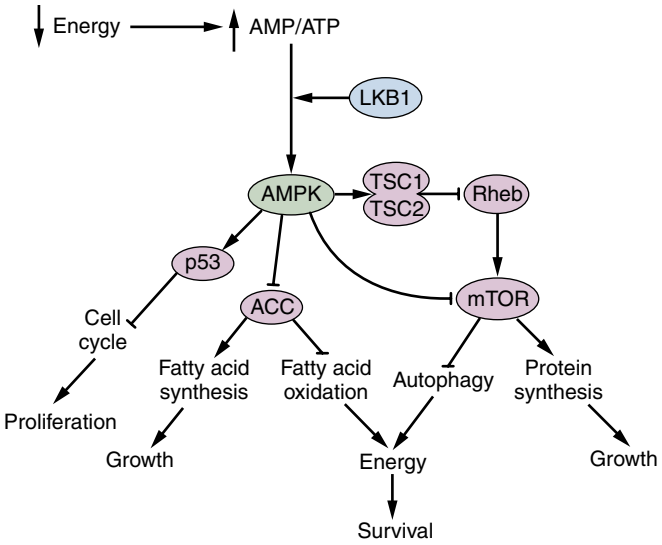


Figure 13-10 The LKB1/AMPK pathway coordinates the metabolic response to nutrient deprivation. A fall in cellular energy status, triggered by nutrient deprivation or other stressors, may increase the adenosine monophosphate (AMP):adenosine triphosphate (ATP) ratio. This activates the kinase KLB1 and its target, the AMP-activated protein kinase (AMPK). AMPK phosphorylates a number of targets to coordinately inhibit anabolic processes and activate catabolic, energy-generating pathways to restore cellular energy levels. In particular, AMPK triggers the phosphorylation and activation of p53, inducing cell cycle arrest; phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), which suppresses fatty acid synthesis and promotes fatty acid oxidation, and direct and indirect inhibition of the mechanistic target of rapamycin (mTOR), thereby inhibiting protein synthesis and inducing autophagy. Suppression of growth and proliferation preserve existing energy stores, while fatty acid oxidation and autophagy generate ATP.

syndrome characterized by benign gastrointestinal and oral lesions and heightened susceptibility to a variety of cancers. Similarly, inactivating mutations in LKB1 are frequent in lung and ovarian carcinomas.[39] The pathogenic mechanisms of LKB1 loss of function are unclear, as LKB1 targets proteins involved in apoptosis, cell polarity, and cell cycle control in addition to activating AMPK. However, it is possible that failure to curtail cell growth in response to normal environmental cues contributes to the oncogenic potential of LKB1 inhibition. In line with this hypothesis, there is great interest in determining whether activating AMPK through agonists such as metformin could activate metabolic checkpoints to block tumor growth. Given the role of AMPK antagonizing many of the pro-growth functions of the PI3K pathway, such strategies may provide an effective way to harness our normal homeostatic mechanisms to slow cancer growth.

The Transcription Factor HIF1 Controls Glycolytic Metabolism

Conditions of low oxygen availability pose a threat to cell survival. Oxygen serves as the terminal electron acceptor of the electron transport chain; as a result, when oxygen is limiting, ATP production through oxidative phosphorylation can be compromised. Excess electrons leak out of the electron transport chain inappropriately, forming reactive oxygen species than can, in high doses, cause great cellular damage. Consequently, cells must rapidly sense and respond to conditions of low oxygen. The cellular response to low oxygen is coordinated by the hypoxia inducible factors (HIF1 and HIF2), transcription factors that are rapidly stabilized and activated during hypoxia. HIF1 and HIF2 promote survival in hypoxia through two mechanisms. First, they induce expression of angiogenic genes, such as vascular endothelial growth factor (VEGF), in order to increase the blood supply to reoxygenate the tissue. Second, they promote metabolic reprogramming in order to maintain ATP supplies while limiting damaging electron loss from the electron transport chain.[40]

Many of the activities of HIF1 and HIF2 would benefit tumor cell survival. As discussed earlier, tumors have frequent variations in oxygenation, often resulting in regions of hypoxia. Expression of HIF1 or HIF2 would help a tumor cell adapt and survive in a hypoxic microenvironment. In particular, HIF1 induces multiple metabolic changes that characterize cancer cells. HIF1 increases expression of glucose transporters and glycolytic enzymes, thereby elevating glucose consumption and glycolytic flux.

Many tumors display aberrant HIF1 expression. Most famously, von Hippel–Lindau disease, characterized by hemangioblastomas of the central nervous system, pheochromocytoma, and renal cell carcinomas, is caused by loss-of-function mutations of the von Hippel–Lindau (VHL) ubiquitin ligase that negatively regulates the stability of HIF1 and HIF2. Tumors exhibit high levels of HIF1, even under normoxia, and constitutively high levels of HIF1 targets. Elevated HIF1 is observed in several solid tumors outside of VHL disease, including breast, ovarian, colon, pancreas, and prostate.[41] In several cases, levels of HIF1 correlate with tumor aggressiveness and poor prognosis. Multiple mechanisms result in increased HIF1 expression: Variable oxygenation, ROS production, oncogene activation, and growth factor signaling (including PI3K and mTOR signaling) can all contribute to heightened HIF1 activity.

Although HIF1 increases glycolysis and lactate production, its effects on metabolism are not uniformly protumorigenic. First, hypoxia causes a decrease in protein synthesis rates—up to 50% of the rates in normal cells—and this decline in protein synthesis is mediated at least in part by repression of mTOR by HIF1.[42] Second, while HIF1 promotes glycolysis, it blocks the mitochondrial oxidation of glucose-derived pyruvate. HIF1 induces pyruvate dehydrogenase kinase (PDK), which inhibits PDH, the enzyme responsible for converting pyruvate to acetyl-CoA. HIF1 therefore reduces the amount of acetyl-CoA that is available for lipid synthesis, instead diverting pyruvate to lactate

for secretion.[43] Although HIF1 expression is sufficient to drive citrate production through reductive carboxylation of glutamine-derived α-ketoglutarate, it is not clear whether this citrate can fully compensate for reduced conversion of pyruvate to acetyl-CoA.[33] Thus, the effects of HIF1 on tumor metabolism are complex. During conditions of true hypoxia, HIF1 expression promotes metabolic adaptations that support tumor survival and increases angiogenesis to support tumor growth. However, during conditions of adequate oxygenation, persistent HIF1 expression would serve to reduce protein and lipid synthesis and hamper cell proliferation. These disparate effects of HIF1 on tumor growth could explain why HIF1 expression is associated with a tumor-suppressive function under certain circumstances.[44]

c-Myc Coordinates Cellular Proliferation and Metabolism

The Myc family of transcription factors (c-Myc, L-Myc, and N-Myc) both stimulates and represses transcription to regulate a vast array of cellular processes, including cell growth and cell cycle progression, and contributes to a variety of human tumors. Myc regulates the expression of an enormous number of genes and consequently can influence processes as diverse as differentiation, vasculogenesis, cell adhesion, cell growth, apoptosis, and DNA damage responses.[45] Although all of these outcomes could contribute significantly to the oncogenic effects of Myc expression, this section focuses on the potential role of Myc in coordinating cell metabolism to support cell proliferation.

Myc heterodimerizes with its binding partner Max to regulate expression of a large number of genes. In particular, c-Myc promotes expression of several cyclins and CDK4 while repressing the cyclin-dependent kinase inhibitors p21 and p27, thus promoting entry into S phase.[45] At the same time, Myc directly reprograms metabolism by increasing expression of genes involved in glycolysis, glutamine metabolism, and mitochondrial biogenesis.[46] c-Myc also targets a number of genes involved in nucleotide biosynthesis, ensuring that cells have the raw material to successfully complete S phase.[47] An extensive survey of Myc targets in a variety of cell lines revealed that genes involved in ribosome biogenesis form a large component of the core Myc signature, further highlighting Myc's function in regulating biomass accumulation.[48] Together, these findings demonstrate that c-Myc functions to upregulate energy production and biosynthetic processes that are required for successful cell replication, thus directly coordinating proliferative metabolism and cell cycle progression.

Myc is a powerful oncogene whose activity is pathologically enhanced in many human cancers; indeed, *MYC* is one of the most highly amplified oncogenes across all types of human cancers.[49] In other cases, chromosomal translocations or increased transcription downstream of hyperactive Wnt signaling contribute to oncogenic activation of Myc.[50] Myc has many activities that could contribute to its ability to promote tumor establishment and maintenance. In some cases, the metabolic reprogramming driven by Myc expression provides critical support for tumor growth. Myc-overexpressing cells are extremely sensitive to nutrient depletion. In particular, cells with high levels of Myc are glutamine addicted, using glutamine as a critical source of TCA cycle metabolites to support rapid cell growth. Consequently, removal of glutamine from the extracellular medium triggers apoptosis in Myc-overexpressing cells. Likewise, blocking conversion of glutamine to α-ketoglutarate through inhibition of glutaminase can block cellular transformation and tumor growth.[51,52]

Mutations in Metabolic Enzymes Can Influence Tumorigenesis

Although the bulk of cancer-associated metabolic rearrangements are directed by signaling events, cellular signaling pathways are not unidirectional. Metabolites themselves can serve as intracellular cues to regulate signaling proteins and influence gene expression. Activation of AMPK by AMP/ATP and mTOR by amino acid levels are common examples of how metabolites can inform signaling pathways. As with canonical signaling pathways, dysregulated activation of these metabolic signaling pathways can influence cancer susceptibility and tumor growth. Mutations in three TCA-cycle enzymes provide major examples of how inappropriate metabolite accumulation can trigger oncogenic signaling events.

Loss-of-function mutations in two consecutive enzymes of the TCA cycle, succinate dehydrogenase (SDH) and fumarate hydratase (FH), predispose patients to tumor formation. In both cases, the genes behave as classic tumor suppressors, in which mutation in one allele causes a dominant risk of tumor formation, followed by loss of function of the second, wild-type allele, in the tumor itself. SDH has four subunits and an assembly factor, and mutation in any of these five genes predisposes individuals to a variety of paragangliomas and pheochromocytomas, and in some cases, renal cell carcinomas or gastrointestinal stromal tumors.[53] In all cases, the pathogenic mechanisms linking SDH deficiency to tumor formation are attributed to elevated succinate levels, triggering a pseudohypoxic cellular state. In normoxia, HIF1 activity is repressed by the action of a family of prolyl hydroxylases (PHDs) that target the labile subunit of HIF1 (HIF1α) for ubiquitination and degradation by the proteasome. PHDs use oxygen, α-ketoglutarate, and ferrous iron to hydroxylate HIF1α, releasing carbon dioxide, succinate, and ferric iron. Consequently, accumulation of succinate blocks PHD activity, resulting in

aberrant stabilization of HIF1α.[54] Enhanced HIF1 activity is likely an important mechanism driving SDH-deficient tumor growth, as the clinical presentations of patients with dominant SDH mutations are similar to several hypoxia syndromes.[53]

Fumarate, likewise, is a potent inhibitor of the PHDs.[55,56] However, FH defects are associated with a predisposition to hereditary leiomyomatosis and renal cell carcinoma.[57] Although elevated HIF1 may contribute to the pathogenic mechanism of FH mutations, several additional consequences of elevated intracellular fumarate provide alternative avenues through which FH mutations may increase cancer susceptibility. First, fumarate can directly modify specific cysteine residues in Kelch-like ECH-associated protein 1 (KEAP1), thereby blocking its ability to suppress the Nrf2-mediated antioxidant response pathway.[58] Enhanced antioxidant defenses may, in some contexts, help guard cancer cell survival in the face of high proliferation-induced ROS production. Other studies have demonstrated that FH deficiency triggers a shift toward glycolytic metabolism, accompanied by reduced AMPK activity, lower intracellular iron, and elevated HIF1.[59] Regardless of the mechanism of tumorigenesis, both SDH- and FH-deficient tumors have the striking phenotype of interrupted TCA cycle function. Elevated aerobic glycolysis can increase glucose consumption and alleviate the bioenergetic constraints. In some cases, mitochondria may be able to funnel accumulated metabolites into alternate metabolic pathways, enabling partial mitochondrial NADH generation.[60] Similarly, glutamine-dependent reductive carboxylation can maintain citrate pools in cells with defective oxidative phosphorylation.[35] Given the critical role of the TCA cycle in supporting anaplerotic reactions, it will be important for future studies to clarify how cells with impaired TCA cycle activities can proliferate at pathological rates.

More recently, the discovery of mutations in the cytosolic and mitochondrial forms of isocitrate dehydrogenase (IDH) has provided the strongest evidence that altered metabolism can drive tumorigenesis. Somatic mutations at a defined number of residues on IDH occur at a high frequency in a variety of malignancies, most notably glioblastoma and acute myeloid leukemia. Oncogenic mutations in IDH confer a neomorphic activity: Rather than oxidizing isocitrate to α-ketoglutarate, mutant IDH isoforms reduce α-ketoglutarate, forming 2-hydroxyglutarate (2-HG).[61,62] A series of studies demonstrated that 2-HG, by virtue of its structural similarity to α-ketoglutarate, can act as an inhibitor of multiple α-ketoglutarate–dependent dioxygenases. In particular, 2-HG can inhibit the TET family of enzymes that convert 5-methylcytosine to 5-hydroxymethylcytosine, an intermediate step in the process of DNA demethylation.[63-65] Likewise, 2-HG can inhibit various histone demethylases in vitro, and mutant IDH expression correlates with increased H3K9 histone methylation in vivo.[65-67] In both cases, hypermethylation profiles (of DNA and/or histones) contribute to the silencing of differentiation-related genes, promoting the accumulation of cells arrested in the course of their differentiation, capable of self-renewal and prone to tumorigenesis.[68]

Together, these studies underscore the complex interplay between cellular signaling networks and metabolic pathways. In all cases, oncogenic activity is attributed to altered levels of particular metabolites (fumarate, succinate, or 2-HG), indicating that cells are highly responsive to alterations in metabolic enzymes and highlighting the possibility that metabolites themselves can function as potent signaling molecules.

Clinical Implications of Metabolic Transformation

The ultimate goal of cancer research is to improve cancer detection and treatment. Elucidating the differences between cancer and normal cells will provide a means to identify and attack cancer cells specifically. In this regard, the distinctive metabolic phenotype of cancer cells may provide a key therapeutic window to distinguish pathological proliferation.

The propensity of tumors to take up high levels of glucose is already extensively exploited in the clinic. Positron emission tomography (PET) enables in vivo visualization of radioactive tracers such as [^{18}F]fluorodeoxyglucose (FDG), a glucose analog that can be taken up by cells and phosphorylated by hexokinase but not further metabolized. Cellular accumulation of FDG therefore provides a reliable readout of the glycolytic activity of a cell, and whole-body scanning by PET can reveal regions of abnormally high glucose uptake and retention. Because cancer cells often take up high levels of glucose, FDG-PET provides a robust tool for detecting a variety of tumors, including breast, colorectal, lung, brain, and lymphoma. FDG-PET can also be used to monitor tumor growth and metastases. Importantly, FDG-PET provides a rapid readout of tumor response to therapy. Most therapies that effectively impair tumor growth or survival trigger a rapid decrease in FDG-PET signal, a change that can be observed within a few days after therapy initiation.[69,70] Because cancer cells often have aberrant nutrient uptake, future studies may reveal other radioactive metabolic tracers with tumor-specific uptake.

Tumor dependence on glycolysis provides additional therapeutic avenues. Promising preclinical data exist for the inhibition of multiple steps of glucose uptake and catabolism through glycolysis. One large caveat in the targeting of metabolic enzymes is that cancer cells often have a metabolic

profile very similar to rapidly proliferating normal cells. Consequently, blocking enzymes that promote proliferative metabolism may have adverse effects on multiple cell types in vivo, including the rapidly proliferating cells in the intestinal epithelium and bone marrow. Nevertheless, it may be possible to safely target glucose metabolism in patients. As discussed earlier, the rate of entry of pyruvate into the TCA cycle is determined by pyruvate dehydrogenase, which is inhibited by the PDKs. Dichloroacetate (DCA) inhibits the PDKs, thus increasing pyruvate flux into the TCA cycle and reducing lactate production. Although the therapeutic benefit of increasing glucose oxidation is not yet demonstrated in tumors in vivo, DCA is well tolerated in patients, suggesting that it may be possible to target aberrant glucose metabolism in cancer patients.[71]

The therapeutic potential of many of the pathways discussed in this chapter are targets of ongoing studies. In animal studies, inhibitors of several steps of glycolysis (phosphofructokinase,[72] lactate dehydrogenase[23,73]), glutamine anaplerosis (glutaminase,[51] glutamate dehydrogenase[74]), and fatty acid synthesis (ATP citrate lyase,[29] fatty acid synthase[75,76]) can inhibit tumor growth. These data suggest that carefully targeted administration of agents that block key nodes of proliferative metabolism may hold therapeutic promise.

Suggestively, some of the oldest and most effective cancer treatments target aspects of cell metabolism. For example, antifolates interfere with folate metabolism and thus disrupt nucleotide biosynthesis and other reactions that require one-carbon units, such as protein methylation. 5-Fluorouracil, a nucleoside analog, inhibits thymidylate synthase and blocks DNA synthesis. These antimetabolites are still effective therapies today, reinforcing the critical role of altered metabolism in the support of tumor growth. Further research into the specific metabolic abnormalities of cancer cells, and how these perturbations foster tumor growth, may uncover pharmacologic targets that will be effective and well tolerated.

Key References

1. Vander Heiden MG, Cantley LC, Thomspon CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324:1029-1033.
2. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature*. 2012;491:364-373.
3. Wallace DC. Mitochondria and cancer. *Nat Rev Cancer*. 2012;12:685-698.
4. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet*. 2005;39:359-407.
5. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci*. 2010;35:427-433.
7. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11:85-95.
8. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*. 2011;27:441-464.
9. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. 2012;21:297-308.
10. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
12. Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E. Energy metabolism in tumor cells. *FEBS J*. 2007;274:1393-1418.
18. Chowdhury SK, Gemin A, Singh G. High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer cell lines. *Biochem Biophys Res Commun*. 2005;333:1139-1145.
19. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*. 2004;4:891-899.
20. Gatenby RA, Gawlinski ET, Gmitro AF, et al. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res*. 2006;66:5216-5223.
21. Robey IF, Baggett BK, Kirkpatrick ND, et al. Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer Res*. 2009;69:2260-2268.
22. Sonveaux P, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest*. 2008;118:3930-3942.
23. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. 2006;9:425-434.
24. Locasale JW, Grassian AR, Melman T, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat Genet*. 2011;43:869-874.
25. Possemato R, Marks KM, Shaul YD, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature*. 2011;476:346-350.
26. Zhang WC, Shyh-Chang N, Yang H, et al. Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell*. 2012;148:259-272.
27. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin Cancer Biol*. 2005;15:300-308.
28. DeBerardinis RJ, Mancuso A, Daikhin E, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A*. 2007;104:19345-19350.
29. Hatzivassiliou G, Zhao F, Bauer DE, et al. ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell*. 2005;8:311-321.
30. Bauer DE, Hatzivassiliou G, Zhao F, et al. ATP citrate lyase is an important component of cell growth and transformation. *Oncogene*. 2005;24:6314-6322.
31. Wise DR, DeBerardinis RJ, Mancuso A, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008;105:18782-18787.
32. Yuneva M, Zamboni N, Oefner P, et al. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol*. 2007;178:93-105.
33. Wise DR, Ward PS, Shay JE, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci U S A*. 2011;108:19611-19616.
34. Metallo CM, Gameiro PA, Bell EL, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. 2012;481:380-384.
35. Mullen AR, Wheaton WW, Jin ES, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*. 2012;481:385-388.
36. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
37. Yecies JL, Manning BD. Transcriptional control of cellular metabolism by mTOR signaling. *Cancer Res*. 2011;71:2815-2820.
38. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
39. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer*. 2009;9:563-575.
40. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399-408.

41. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*. 2010;29:625-634.
42. Spriggs KA, Bushell M, Willis AE. Translational regulation of gene expression during conditions of cell stress. *Mol Cell*. 2010;40:228-237.
43. Lum JJ, Bui T, Gruber M. The transcription factor HIF-1alpha plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev*. 2007;21:1037-1049.
44. Shen C, Beroukhim R, Schumacher SE, et al. Genetic and functional studies implicate HIF1alpha as a 14q kidney cancer suppressor gene. *Cancer Discov*. 2011;1:222-235.
45. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6:635-645.
46. Dang CV, Kim JW, Gao P, Yustein J. The interplay between MYC and HIF in cancer. *Nat Rev Cancer*. 2008;8:51-56.
47. Liu YC, Li F, Handler J, et al. Global regulation of nucleotide biosynthetic genes by c-Myc. *PLoS One*. 2008;3:e2722.
48. Ji H, Wu G, Zhan X, et al. Cell-type independent MYC target genes reveal a primordial signature involved in biomass accumulation. *PLoS One*. 2011;6:e26057.
49. Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
50. Dang CV. MYC on the path to cancer. *Cell*. 2012;149:22-35.
51. Wang JB, Erickson JW, Fuji R, et al. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell*. 2010;18:207-219.
52. Le A, Lane AN, Hamaker M, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab*. 2012;15:110-121.
53. Raimundo N, Baysal BE, Shadel GS. Revisiting the TCA cycle: signaling to tumor formation. *Trends Mol Med*. 2011;17:641-649.
54. Selak MA, Armour SM, MacKenzie ED, et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell*. 2005;7:77-85.
55. Iaacs JS, Jung YJ, Mole DR, et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell*. 2005;8:143-153.
56. MacKenzie ED, et al. Cell-permeating alpha-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. *Mol Cell Biol*. 2007;27:3282-3289.
58. Adam J, Hatipoglu E, O'Flaherty L, et al. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell*. 2011;20:524-537.
59. Tong WH, Sourbier C, Kovtunovych G, et al. The glycolytic shift in fumarate-hydratase-deficient kidney cancer lowers AMPK levels, increases anabolic propensities and lowers cellular iron levels. *Cancer Cell*. 2011;20:315-327.
60. Frezza C, Zheng L, Folger O, et al. Haem oxygenase is synthetically lethal with the tumour suppressor fumarate hydratase. *Nature*. 2011;477:225-228.
61. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*. 2009;462:739-744.
62. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell*. 2010;17:225-234.
63. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553-567.
64. Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature*. 2012;483:479-483.
65. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell*. 2011;19:17-30.
66. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature*. 2012;483:474-478.
67. Chowdhury R, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. *EMBO Rep*. 2011;12:463-469.
68. Lu C, Thompson CB. Metabolic regulation of epigenetics. *Cell Metab*. 2012;16:9-17.
69. Engelman JA, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med*. 2008;14:1351-1356.
71. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nat Rev Drug Discov*. 2011;10:671-684.
72. Clem B, Telang S, Clem A, et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol Cancer Ther*. 2008;7:110-120.
74. Yang C, Sudderth J, Dang T, et al. Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res*. 2009;69:7986-7993.
76. Flavin R, Peluso S, Nguyen PL, Loda M. Fatty acid synthase as a potential therapeutic target in cancer. *Future Oncol*. 2010;6:551-562.

This page intentionally left blank

References

1. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009;324:1029-1033.
2. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature*. 2012;491:364-373.
3. Wallace DC. Mitochondria and cancer. *Nat Rev Cancer*. 2012;12:685-698.
4. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet*. 2005;39:359-407.
5. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci*. 2010;35:427-433.
6. Berg JM, et al. *Biochemistry*. New York, NY: W.H. Freeman; 2002.
7. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nat Rev Cancer*. 2011;11:85-95.
8. Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*. 2011;27:441-464.
9. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. 2012;21:297-308.
10. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
11. Warburg O. On the origin of cancer cells. *Science*. 1956;123:309-314.
12. Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E. Energy metabolism in tumor cells. *FEBS J*. 2007;274:1393-1418.
13. Warburg O. On respiratory impairment in cancer cells. *Science*. 1956;124:269-270.
14. Guppy M, Greiner E, Brand K. The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes. *Eur J Biochem*. 1993;212:95-99.
15. MacDonald MJ, et al. High activity of mitochondrial glycerol phosphate dehydrogenase in insulinomas and carcinoid and other tumors of the amine precursor uptake decarboxylation system. *Cancer Res*. 1990;50:7203-7205.
16. Grivell AR, Korpelainen EI, Williams CJ, Berry MN. Substrate-dependent utilization of the glycerol 3-phosphate or malate/aspartate redox shuttles by Ehrlich ascites cells. *Biochem J*. 1995;310(Pt 2):665-671.
17. Mazurek S, Eigenbrodt E, Failing K, Steinberg P. Alterations in the glycolytic and glutaminolytic pathways after malignant transformation of rat liver oval cells. *J Cell Physiol*. 1999;181:136-146.
18. Chowdhury SK, Gemin A, Singh G. High activity of mitochondrial glycerophosphate dehydrogenase and glycerophosphate-dependent ROS production in prostate cancer cell lines. *Biochem Biophys Res Commun*. 2005;333:1139-1145.
19. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*. 2004;4:891-899.
20. Gatenby RA, Gawlinski ET, Gmitro AF, et al. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res*. 2006;66:5216-5223.
21. Robey IF, Baggett BK, Kirkpatrick ND, et al. Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer Res*. 2009;69:2260-2268.
22. Sonveaux P, et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest*. 2008;118:3930-3942.
23. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. 2006;9:425-434.
24. Locasale JW, Grassian AR, Melman T, et al. Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat Genet*. 2011;43:869-874.
25. Possemato R, Marks KM, Shaul YD, et al. Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature*. 2011;476:346-350.
26. Zhang WC, Shyh-Chang N, Yang H, et al. Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. *Cell*. 2012;148:259-272.
27. Mazurek S, Boschek CB, Hugo F, Eigenbrodt E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin Cancer Biol*. 2005;15:300-308.
28. DeBerardinis RJ, Mancuso A, Daikhin E, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A*. 2007;104:19345-19350.
29. Hatzivassiliou G, Zhao F, Bauer DE, et al. ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell*. 2005;8:311-321.
30. Bauer DE, Hatzivassiliou G, Zhao F, et al. ATP citrate lyase is an important component of cell growth and transformation. *Oncogene*. 2005;24:6314-6322.
31. Wise DR, DeBerardinis RJ, Mancuso A, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc Natl Acad Sci U S A*. 2008;105:18782-18787.
32. Yuneva M, Zamboni N, Oefner P, et al. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol*. 2007;178:93-105.
33. Wise DR, Ward PS, Shay JE, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci U S A*. 2011;108:19611-19616.
34. Metallo CM, Gameiro PA, Bell EL, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. 2012;481:380-384.
35. Mullen AR, Wheaton WW, Jin ES, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*. 2012;481:385-388.
36. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
37. Yecies JL, Manning BD. Transcriptional control of cellular metabolism by mTOR signaling. *Cancer Res*. 2011;71:2815-2820.
38. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
39. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer*. 2009;9:563-575.
40. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399-408.
41. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene*. 2010;29:625-634.
42. Spriggs KA, Bushell M, Willis AE. Translational regulation of gene expression during conditions of cell stress. *Mol Cell*. 2010;40:228-237.
43. Lun JJ, Bui T, Gruber M. The transcription factor HIF-1alpha plays a critical role in the growth factor-dependent regulation of both aerobic and anaerobic glycolysis. *Genes Dev*. 2007;21:1037-1049.
44. Shen C, Beroukhim R, Schumacher SE, et al. Genetic and functional studies implicate HIF1alpha as a 14q kidney cancer suppressor gene. *Cancer Discov*. 2011;1:222-235.
45. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6:635-645.
46. Dang CV, Kim JW, Gao P, Yustein J. The interplay between MYC and HIF in cancer. *Nat Rev Cancer*. 2008;8:51-56.
47. Liu YC, Li F, Handler J, et al. Global regulation of nucleotide biosynthetic genes by c-Myc. *PLoS One*. 2008;3:e2722.
48. Ji H, Wu G, Zhan X, et al. Cell-type independent MYC target genes reveal a primordial signature involved in biomass accumulation. *PLoS One*. 2011;6:e26057.
49. Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
50. Dang CV. MYC on the path to cancer. *Cell*. 2012;149:22-35.
51. Wang JB, Erickson JW, Fuji R, et al. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell*. 2010;18:207-219.
52. Le A, Lane AN, Hamaker M, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab*. 2012;15:110-121.
53. Raimundo N, Baysal BE, Shadel GS. Revisiting the TCA cycle: signaling to tumor formation. *Trends Mol Med*. 2011;17:641-649.
54. Selak MA, Armour SM, MacKenzie ED, et al. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell*. 2005;7:77-85.
55. Isaacs JS, Jung YJ, Mole DR, et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell*. 2005;8:143-153.
56. MacKenzie ED, et al. Cell-permeating alpha-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. *Mol Cell Biol*. 2007;27:3282-3289.

57. Tomlinson IP, Alam NA, Rowan AJ, et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat Genet*. 2002;30:406-410.
58. Adam J, Hatipoglu E, O'Flaherty L, et al. Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. *Cancer Cell*. 2011;20:524-537.
59. Tong WH, Sourbier C, Kovtunovych G, et al. The glycolytic shift in fumarate-hydratase-deficient kidney cancer lowers AMPK levels, increases anabolic propensities and lowers cellular iron levels. *Cancer Cell*. 2011;20:315-327.
60. Frezza C, Zheng L, Folger O, et al. Haem oxygenase is synthetically lethal with the tumour suppressor fumarate hydratase. *Nature*. 2011;477:225-228.
61. Dang L, White DW, Gross S, et al. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*. 2009;462:739-744.
62. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell*. 2010;17:225-234.
63. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell*. 2010;18:553-567.
64. Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature*. 2012;483:479-483.
65. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell*. 2011;19:17-30.
66. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature*. 2012;483:474-478.
67. Chowdhury R, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. *EMBO Rep*. 2011;12:463-469.
68. Lu C, Thompson CB. Metabolic regulation of epigenetics. *Cell Metab*. 2012;16:9-17.
69. Engelman JA, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med*. 2008;14:1351-1356.
70. Young H, et al. Measurement of clinical and subclinical tumour response using [18F]-fluorodeoxyglucose and positron emission tomography: review and 1999 EORTC recommendations. European Organization for Research and Treatment of Cancer (EORTC) PET Study Group. *Eur J Cancer*. 1999;35:1773-1782.
71. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nat Rev Drug Discov*. 2011;10:671-684.
72. Clem B, Telang S, Clem A, et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol Cancer Ther*. 2008;7:110-120.
73. Shim H, Dolde C, Lewis BC, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A*. 1997;94:6658-6663.
74. Yang C, Sudderth J, Dang T, et al. Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res*. 2009;69:7986-7993.
75. Pizer RD, Wood FD, Heine HS, et al. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. *Cancer Res*. 1996;56:1189-1193.
76. Flavin R, Peluso S, Nguyen PL, Loda M. Fatty acid synthase as a potential therapeutic target in cancer. *Future Oncol*. 2010;6:551-562.

Eileen White, Douglas R. Green, and Anthony G. Letai

14

Apoptosis, Necrosis, and Autophagy

In the simple arithmetic of life, a tissue grows if cells divide more frequently than they die, whereas one in which cell death is more frequent than cell division shrinks. This arithmetic, trivial as it seems, is at the heart of the understanding of cancer and the efforts to effectively treat it. However, the mechanisms that control this relationship are far from trivial.

One theory of cancer, in fact, suggests that an evolutionary "hard-wiring" of the machineries of tissue expansion and cell death is fundamental to tumor suppression. This concept, called *antagonistic pleiotropy* (Figure 14-1),[1] states that *any* molecular event in a multicellular organism that directs a cell to divide also instructs it to die, and that the latter will occur unless additional signals (either exogenous or endogenous) promote cell survival.

It is critical to note that this idea is fundamentally different from the prevalent (and incorrect) notion that "cancer cells are resistant to cell death," which as we will see is not only inaccurate, but patently untrue. Antagonistic pleiotropy only demands that the *specific* cell death pathways engaged by signals promoting clonal expansion be sufficiently muted that the rate of division exceeds the rate of cell death. Other pathways of cell death not only remain available, but may be sensitized in the process, providing us with the opportunity to shift the balance by therapies.

The Ways Cells Die

There are different ways in which cells die, and these are grouped on the basis of the morphologies of the dying cells (Figure 14-2)[2] and more recently by the mechanisms of cell death as we understand them.[3] By general agreement, there are three modalities of cell death: apoptosis (type I), autophagic cell death (type II), and necrosis (type III). Within each of these modes there are additional varieties of morphologies and/or pathways that engage death.

Apoptosis is characterized by condensed chromatin, membrane blebbing, and cell shrinkage, and the cell often breaks into smaller, membrane-bound bodies (see Figure 14-2). The DNA within chromatin is cleaved by the action of a nuclease that cuts the DNA between nucleosomes, producing the degraded DNA hallmark of apoptosis. Before the loss of plasma membrane integrity, the lipids of the membrane "scramble," resulting in the appearance of phospholipids on the surface, which are normally preferentially associated with the inner leaflet. All of these events are due to the action of caspase proteases that are activated during apoptosis. Usually, apoptotic cells are effectively cleared by phagocytosis ("cell eating") by macrophages and other cell types, before the contents of the dying cell can be released. This involves the recognition of a phospholipid, phosphatidylserine, which appears on the outer leaflet as a consequence of the scrambling event just noted. As a result, apoptotic cell death does not usually engage an inflammatory response and is sometimes described as being immunologically "silent."

Necrosis, in contrast, involves swelling of the cell and organelles. As plasma membrane integrity is disrupted and the contents of the cell spill out, some components act as signals to other cells that damage has occurred (see Figure 14-2). These signals, called *damage-associated molecular patterns* (DAMPs), can trigger inflammatory responses.

The third mode of cell death, autophagic cell death, is problematic. It is characterized by the formation of large vacuoles (often enlarged lysosomes) and the activation of autophagy (see Figure 14-2). As with necrosis, caspases are not activated. However, there is considerable disagreement regarding the role of autophagy in the cell death itself. In most cases, disruption of the autophagy machinery accelerates the cell death, and therefore this is viewed as "cell death accompanied by autophagy."[2] There are examples in *Drosophila*, however, in which the autophagic machinery is fundamentally involved in the cell death process itself,[4] although it is unclear to what extent such bona fide autophagic cell death occurs in mammals.

In thinking about the different modes of cell death, a useful distinction can be made between cell death that is "passive," meaning that the cell is killed, and that which is "active," that is, the cell participates in its own demise. In the first case, cell death can only be prevented by removing or blocking the toxic insult, or by repairing damage more quickly than it occurs. In cases of active cell death, this quietus may also be blocked if the cellular pathways involved are inhibited or disrupted.

It is important to note, however, that just because a cell death process is active, this does not mean that it represents a specialized cell death mechanism that was evolutionarily selected as a physiological pathway of cell death. Much as a running but not a motionless train can be destroyed by derailing, a cellular activity can be "sabotaged" to inflict lethal damage on the cell. In contrast, there are specialized cell death pathways that are indeed mechanisms of physiological cell death, and thus can be regarded as cellular "suicide."

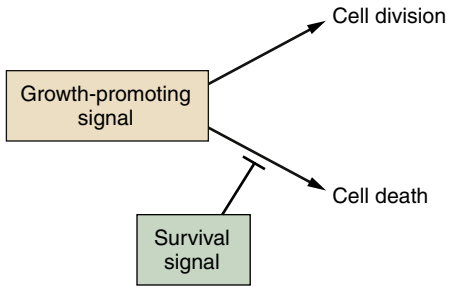


Figure 14-1 Antagonistic pleiotropy This concept states that signals that engage the cell cycle also engage cell death, and tissues therefore expand only if cell death is blocked. Note that this does not mean that the cell cycle, or any component of its machinery, is responsible for cell death. Survival signals can either be cell intrinsic or extrinsic and need only be specific for the death mechanism engaged by the original growth-promoting signal. The term *antagonistic pleiotropy* is borrowed from a theory of aging of the same name, which on the surface is very different from the concept of tumor suppression as used here. There are, however, interesting parallels. In the aging theory, genes that are beneficial early in the life of an animal will be selected, even if their activities are detrimental later in life. The reader might wish to consider how this concept may apply to aging and cancer and how these ideas relate to the use of the term here.

Figure 14-3 illustrates these distinctions, although in many cases we can only speculate as to whether a process is sabotage versus suicide.[5]

From the perspective of cancer biology, however, the distinction is particularly useful. When cell death is engaged as a mechanism of tumor suppression, it may be regarded as suicide, whereas the effects of therapeutic intervention may manifest either by recruiting such a suicide pathway or by effectively sabotaging a process in the cancer cell to result in its death.

The best example of active cell suicide is apoptosis, although there may be others (as we note in Figure 14-3 and in the following discussion). In contrast, the lethal overproduction of reactive oxygen species (ROS) by disruption of the mitochondrial electron transport chain, the engagement of membrane NADH oxidases, or the loss of ROS scavenging mechanisms may be regarded as cellular sabotage (see Figure 14-3, and other examples discussed later).

This distinction blurs when we consider the evolution of cell death. Any mechanism of cellular sabotage could well be selected to become a process of cellular suicide, and indeed, many scenarios for the evolution of apoptosis begin with this premise. At this point, it may be best to remind the

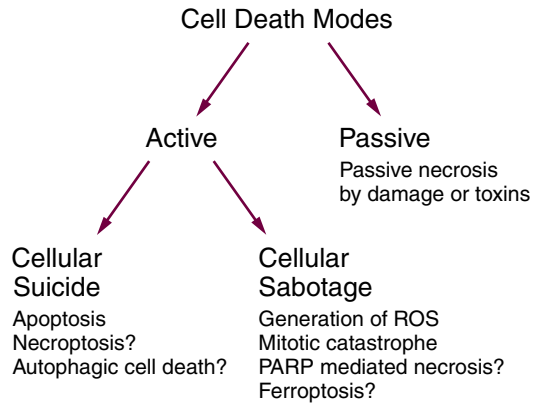


Figure 14-3 Active and passive cell death See text for explanation. *PARP*, polyADP-ribose polymerase; *ROS*, reactive oxygen species.

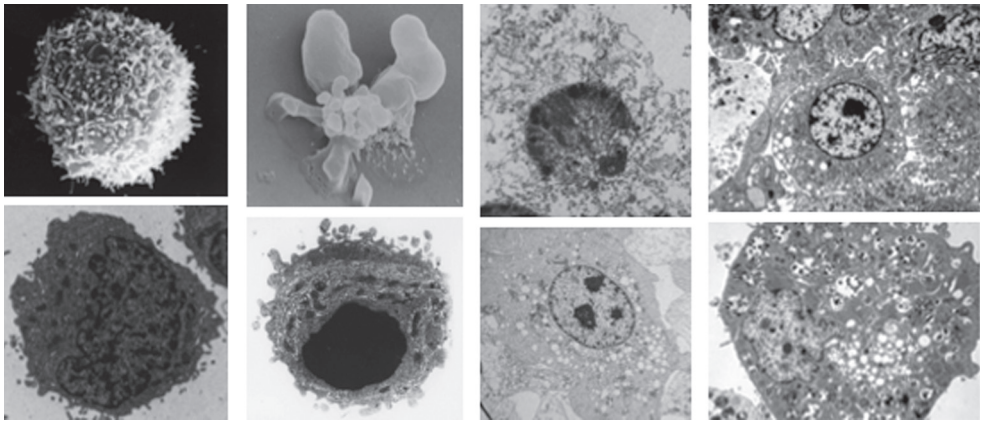


Figure 14-2 Types of cell death Electron micrographs of cell death showing features of each. See text for explanation. *NOTE: All figures except as noted are from DRG. Credits: Living and apoptotic cells, DRG and Yufang Shi; necrotic cells, DRG and Nigel Waterhouse; and autophagic cell death, from Maclean KH, Dorsey FC, Cleveland JL, Kastan MB. Targeting lysosomal degradation induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis.* J Clin Invest. *2008;118:79-88.*

reader that these distinctions are, like many classifications, merely a starting point for understanding the intricacies of the processes. Nevertheless, it might be interesting to think about why, of all the possible cell suicide mechanisms that *might* have evolved, apoptosis has emerged as the predominant form of active cell death in the animal kingdom.

Apoptosis

Caspase Activation

Apoptosis is orchestrated by the actions of caspases, which are cysteine proteases (that is, they have a cysteine at the active sites). There are at least 17 caspases that have been described in mammals, and we understand the functions of only a few of them. The process of apoptosis occurs through the activation of a subset of these, called the *executioner caspases* (caspase-3, caspase-6, and caspase-7 in mammals), and once activated, these cut up to a thousand different cellular substrates in the cell to precipitate the changes associated with this form of cell death. The "cuts" are at specific sites in the substrates and occur at aspartate residues. This is the origin of "caspase," a *c*ysteine protease with *Asp-ase* activity.

The executioner caspases are present in cells in an inactive, dimeric form, which becomes activated when cut at specific aspartate residues, resulting in a conformational change that forms the active enzyme. The active enzyme now cuts its substrates, some of which are responsible for the changes associated with apoptosis.[6]

In general, this activating cleavage is mediated by other caspases, called *initiator caspases*. Unlike the executioner caspases, the inactive forms of the initiator caspases present in cells are monomeric and contain large prodomains that include sites for protein-protein interaction. Adapter molecules, which form "caspase activation platforms," bind to the prodomains of initiator caspases, forcing them into contact, and the latter then fold into active enzymes. These can then process the executioner caspases, thereby activating them to cleave their substrates and precipitate apoptosis.

The formation of caspase activation platforms and the caspases they activate define the apoptotic pathways. Here we consider in detail only two major pathways of apoptosis, the mitochondrial pathway and the death receptor pathway. It should be noted, however, that there are others, reviewed elsewhere.[7] One of these involves the formation of caspase activation platforms for the engagement and activation of caspase-1, which is critically involved in inflammatory responses by processing specific cytokines and promoting their release. The activation of caspase-1 can also kill cells by a process resembling apoptosis (often called *pyroptosis*). The activation of caspase-1 occurs in response to many infectious agents and has been implicated in promoting forms of cancer that are associated with inflammatory conditions. Caspases can directly signal apoptosis or can use mitochondria as an intermediate, and additional point of regulation, in signaling of apoptosis.

The Mitochondrial Pathway of Apoptosis

Starting in the 1980s, the intracellular molecules that controlled the morphology referred to as apoptosis began to be identified.[8,9] Cell death had previously been thought of as a passive event (as noted earlier): a universally negative occurrence that cells were constructed to avoid at all costs. Careful observations in human pathological samples as well as in model systems revealed that instead, cell death in multicellular organisms was often a carefully controlled event with stereotypical morphologic and temporal patterns. Moreover, when it was observed that altering the function of certain genes could alter the commitment, phenotype, and progression to cell death, it became clear that cells contained within them genetic programs to perform a function of choosing death as a cell fate, and then committing cell suicide. Although several different forms of programmed cell death have been identified, the first to be characterized was apoptosis.

The mitochondrial (sometimes called *intrinsic*) pathway of apoptosis responds to a diverse group of initiating events, including treatment with a wide variety of cytotoxic drugs, growth factor withdrawal, and oncogene activation. The mitochondrial pathway of apoptosis get its name because of the centrality of this organelle in coordinating both cell fate decision making as well as execution. Key features of apoptosis via the mitochondrial pathway include mitochondrial outer membrane permeabilization (MOMP), followed by release to the cytosol of molecules that facilitate execution of the death program, including cytochrome c, SMAC, and Omi. Cytochrome c is essential to the formation of one of the caspase activation platforms, the apoptosome, which is formed when cytochrome c activates APAF-1 to oligomerize, and the latter then binds and activates caspase-9.[10] Although caspases may perform normal physiological functions, they are best known for cleavage of proteins during apoptosis important for cell integrity, including cytoskeletal proteins and PARP-1. Among the caspase-dependent phenomena commonly observed in association with apoptosis are extracellular exposure of the phosphatidylserine that is usually found only on the inner leaflet of the plasma membrane (which results in recognition by Annexin V) and internucleosomal cleavage of chromosomal DNA (which results in laddering of DNA on electrophoresis and nuclear condensation). In vivo in a multicellular organism, an important

consequence of these changes is recognition and clearance of the apoptotic cell by phagocytic cells. It may well be that commitment to cell death is already made at the point of MOMP, whereas many of the downstream morphologies we associate with apoptosis might be most important mainly in facilitating the clearance of apoptotic cells, minimizing inflammation, and optimizing cannibalization of their macromolecules.[11-13]

The BCL-2 Protein Family

The B cell leukemia/lymphoma 2 (BCL-2) family of proteins controls commitment to apoptotic cell death via the mitochondrial pathway.[9] The discovery and characterization of this important family is intimately linked to cancer, as BCL-2 was initially cloned from the t(14;18) chromosomal translocation that is present in nearly all cases of follicular lymphoma.[14-16] The translocation places the *bcl-2* gene on chromosome 18 under the control of regulatory elements of immunoglobulin genes, yielding overexpression of the BCL-2 protein in cells of the B-lineage.

The key step in apoptosis commitment that is controlled by the BCL-2 family is MOMP.[17-19] In simplest terms, there are pro- and anti-apoptotic members of the BCL-2 family. When the pro-apoptotic proteins overwhelm the anti-apoptotic ones, MOMP occurs, and the cell is irreversibly committed to apoptosis. The details of this commitment involve complicated interactions among the BCL-2 family proteins that number greater than a dozen. BAX and BAK are obligate pro-apoptotic "effectors," proteins that homo-oligomerize to form the pores that cause MOMP.[20] In order to form the oligomers required to cause MOMP, BAX and BAK must be activated.[21,22] Proteins of the pro-apoptotic "activator" BH3-only protein subfamily, which include BIM, BID, and perhaps PUMA, can directly interact with BAX and BAK to cause an allosteric change that functionally corresponds to activation. There may also be other ways that BAX and BAK are activated, perhaps even independent of interaction with other proteins.[23]

Anti-apoptotic BCL-2 family proteins include BCL-2, BCL-XL, BCL-w, MCL-1, and BFL-1 (A1). These proteins inhibit apoptosis by binding and sequestering activator proteins or monomeric "activator" BAX or BAK proteins before they can oligomerize.[24,25] Another subfamily, the pro-apoptotic "sensitizer" BH3-only proteins, lack the ability to directly activate BAX and BAK with high efficiency, but promote apoptosis by competitively inhibiting the ability of anti-apoptotic proteins to bind activators, BAX, or BAK (Figure 14-4).[21]

BH3-only proteins get their name from their possession of the BCL-2 homology 3 (BH3) region, but of no other BCL-2 homology region.[26] The BH3 domain is essential for the pro-death activity of all of the pro-apoptotic BCL-2 family proteins. It is a roughly 20 amino acid amphipathic α-helix that binds into a hydrophobic BH3 binding cleft present in all of the anti-apoptotic proteins. There is a selective pattern of interaction of BH3-only proteins with anti-apoptotic proteins.[24,27-29] For instance, the BAD BH3 domain interacts selectively with BCL-2, BCL-XL, and BCL-w, whereas the NOXA BH3 peptide interacts selectively with MCL-1. Small-molecule BH3 domain mimetics that compete for these interactions, effectively inhibiting anti-apoptotic protein function, are being tested in cancer clinical trials (see later discussion).[30]

Mitochondrial Priming and Chemotherapy Response

The concept has emerged from many studies, especially in murine models of cancer, that apoptosis serves as a natural barrier to carcinogenesis.[31,32] In several murine models, defects in apoptosis facilitate carcinogenesis, supporting this concept.[33-35] Many of the changes that commonly occur in oncogenesis, including genomic instability and oncogene activation, contribute to pro-apoptotic signaling, which may well result in deletion of nascent malignant clones. Malignant clones that do survive must therefore have selected for mechanisms of evading apoptotic death provoked by this pro-apoptotic signaling by some combination of fostering anti-apoptotic signaling and attenuating pro-apoptotic signaling.

However, as it is commonly used, the oft-made assertion that resistance to apoptosis is a fundamental property of all cancer begs the question, "Compared to what?" It is difficult to find experimental or clinical evidence that cancers generally are more resistant to apoptosis than the normal nonmalignant cells in the human body. The fact that cancer cells have successfully buffered prior pro-apoptotic signaling does not necessarily mean that they are well prepared to evade subsequent pro-apoptotic signaling.

In fact, many conventional chemotherapies used in the clinic kill cells via apoptosis. The specific mechanisms may vary widely. Taxanes may increase levels of BIM, alter subcellular localization of BH3-only proteins, and decrease levels of MCL-1.[36-38] Cytotoxic agents can induce a decrease in MCL-1 levels, though whether this is a necessary and sufficient step for commitment to apoptosis in these cases is less clear.[39] DNA-damaging agents, by activating p53, can activate transcription of pro-apoptotic PUMA, NOXA, and BAX (see also Chapters 3 and 15).[40-43] In addition, p53 may promote apoptosis via direct interaction with BCL-2 family proteins.[44,45] Space does not permit detailing all of the relationships that have been discovered between conventional

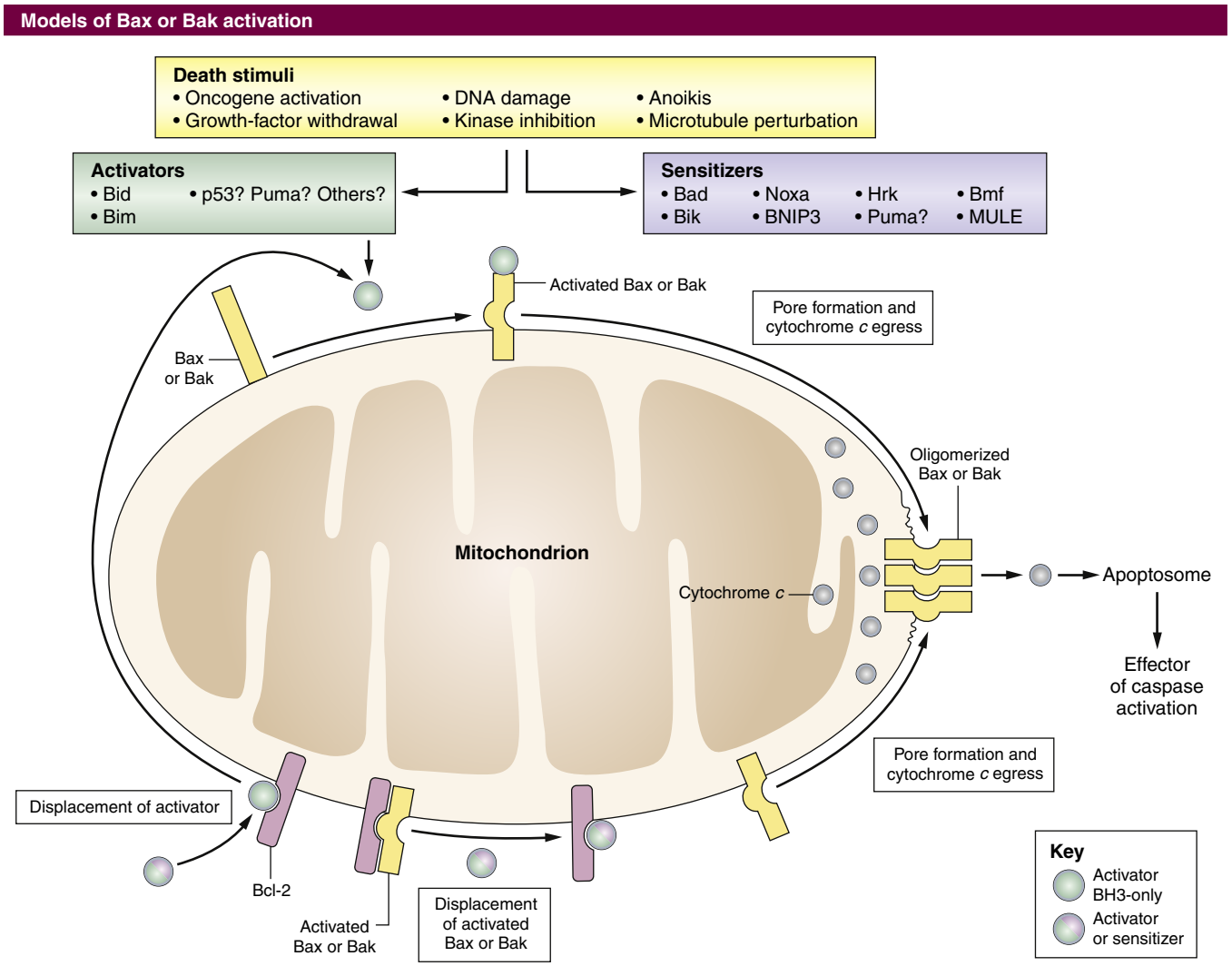


Figure 14-4 Control of MOMP by the BCL-2 family of proteins MOMP occurs following homo-oligomerization of activated BAX and/or BAK into a pore *(right)*, permitting release of proteins such as cytochrome c that facilitate caspase activation. BAX or BAK pore formation can be caused by the displacement of monomeric BAX or BAK that is already activated from anti-apoptotic proteins. This displacement can be effected by BH3-only proteins or BH3 mimetics *(bottom)*. Alternatively, in response to a variety of stimuli, an increase in activator proteins such as BIM or BID can activate BAX or BAK, which can then homo-oligomerize *(top)*. Sensitizer BH3 proteins or mimetics can also displace activators from anti-apoptotic proteins to effect BAX or BAK activation *(left)*.

chemotherapy agents and the BCL-2 family. Although there are sometimes attempts to identify the key BCL-2 family dictating response to a particular agent, it is likely that following most chemotherapies, more than one BCL-2 family protein takes part in determining the cell fate decision.

The ability of chemotherapy to selectively kill cancer cells makes it likely that in many cancer cells the apoptotic pathway is not only intact, but even more sensitive to pro-apoptotic signaling than in normal cells. This hypothesis has been directly tested using a mitochondrial assay called *BH3 profiling*, an assay that systematically compares mitochondrial response to a standardized panel of synthetic BH3 domain peptides.[46,47] These studies have shown that chemosensitive cancer cells are indeed more sensitive to pro-apoptotic signals than normal cells, suggesting mitochondrial apoptotic priming as an important determinant of the therapeutic index in vivo and in vitro. Normal hematopoietic cells are the most primed of normal tissues, consistent with their perennial role as the site of dose-limiting toxicity for most cytotoxic regimens. Measuring pretreatment mitochondrial priming may even be useful as a predictive biomarker, because it correlates well with clinical response.[46,47]

From a perspective of protein biochemistry, cells that are highly primed have little anti-apoptotic reserve to buffer subsequent pro-apoptotic signaling. Very often, a highly primed cell will express abundant anti-apoptotic BCL-2 family proteins, but these will already be occupied by pro-apoptotic activators such as BIM.[24,48,49] In these cases, the cells are dependent on the continuous function of the anti-apoptotic protein(s) for survival. Inhibition of one or more of

these can result in MOMP and apoptosis in highly primed cells (Figure 14-5).

It is worth considering the distinction between expression of an anti-apoptotic protein, and dependence on that anti-apoptotic protein. If one enforces BCL-2 overexpression via transfection of a plasmid vector in a healthy cell line already well established in culture, one will not necessarily obtain a BCL-2–dependent cell line, because there is likely no dependence on the additional BCL-2. The cell line was doing fine without it before transfection, and inhibition of BCL-2 would just restore the status quo ante. In oncogenesis, however, increased expression of BCL-2 is selected for, not extrinsically induced. This implies that the increased BCL-2 that was selected for is required for the continuous buffering of pro-apoptotic signals. In biochemical terms, that means that BCL-2 is largely already in complex with pro-apoptotic BCL-2 family members such as BIM or BAX. In the instance of the transfected cell line, however, BCL-2 is mainly empty and ready to buffer subsequent pro-apoptotic signaling. In the case where BCL-2 expression has been positively selected in the face of extant pro-apoptotic signaling, the BCL-2 is mainly full. Rather than providing anti-apoptotic reserve, the BCL-2 in the latter case is actually storing pro-apoptotic proteins at the mitochondrion, priming it for sensitivity to apoptosis.[24] The cancer cell just discussed is thus dependent on continuous BCL-2 function for survival. In other cancer cells, similar to the transfected cell line, BCL-2 expression may be incidental, rather than selected for, and the BCL-2 not required tonically. Protein and transcript levels of multiple BCL-2 family members, as well as functional assays such as BH3 profiling, have been used to identify cells that are dependent on BCL-2 and hence likely to be sensitive to antagonists of BCL-2 (see later discussion).[48,50,51]

Targeted Therapies and Apoptosis

In the past decade, modern "targeted" therapies have occupied the lion's share of attention in preclinical development, with several achieving approval for clinical use in cancer. Most of these agents also kill by inducing signaling via the mitochondrial apoptotic pathway. However, in this case, selective killing of cancer cells depends less on differences in pretreatment mitochondrial priming and more on selective dependencies of cancer cells. For instance, imatinib, the first small-molecule kinase inhibitor to be approved for use in cancer, inhibits activity of the Abl kinase, an activity that is enhanced by the BCR/ABL fusion protein created by the t(9;22) chromosomal translocation present in chronic myelogenous leukemia (CML). The selective killing of malignant CML cells over normal cells depends on the selective dependence of CML cells on activity of the BCR/ABL kinase.[52] Another family of tyrosine kinase inhibitors, those directed against epidermal growth factor receptor (EGFR), are selectively effective in lung cancers with activating mutations in EGFR.[53-55]

A general property of kinase inhibitors in cancer, whether tyrosine kinase or serine/threonine kinase inhibitors, is their utilization of the mitochondrial apoptotic pathway to kill cancer cells. Interestingly, although doubtless other BCL-2 family members also contribute, BIM, a pro-apoptotic activator BH3-only protein, is regulated by many different kinase inhibitors. In turn, lethality of kinase inhibition depends on the upregulation of BIM. BIM upregulation is required for killing by imatinib whether in CML or in gastrointestinal stromal tumors (GIST) where the constitutively active c-KIT kinase is the relevant target.[56,57] Similar observations have been made for inhibitors of EGFR, MEK, ALK, and b-RAF.[37,58-61] A fascinating human genetic study found that a germline polymorphism of BIM that removes the BH3 domain that is required for pro-apoptotic function is common in an Asian population.[62] Intriguingly, this polymorphism conferred inferior clinical response to inhibitors of both BCR-ABL in CML and of EGFR in non–small-cell lung cancer, apparently confirming its importance in cancer cell–fate decision making in clinical use of kinase inhibitors.

Proteasome inhibitors such as bortezomib have become a mainstay of multiple myeloma therapy and have found application in other diseases such as mantle-cell lymphoma. These also apparently kill via the mitochondrial apoptotic pathway. In this case, the most often cited mechanism of response is the pro-apoptotic BH3-only family protein NOXA, which can selectively bind and inactivate MCL-1, an anti-apoptotic protein also in the BCL-2 family.[63] Of course, proteasome inhibitors alter the levels of thousands of proteins, so that there may be several proteins that are affected that are important for cell-fate decision after proteasome inhibitors.

Directly Targeting the BCL-2 Family in Cancer Treatment

Given the centrality of the BCL-2 family of proteins in determining cell fate in cancer, considerable effort has been expended on inhibition of anti-apoptotic BCL-2 family proteins to induce cancer cell death.[30]

These efforts are in their infancy, and important questions need to be resolved. For example, should the agent have a narrow or broad spectrum of inhibition of anti-apoptotic BCL-2 family proteins? Broad-spectrum agents will likely be more toxic to more kinds of cancers; however, there is the possibility of a narrowed therapeutic index that could limit clinical utility. Will these agents be best used as single agents

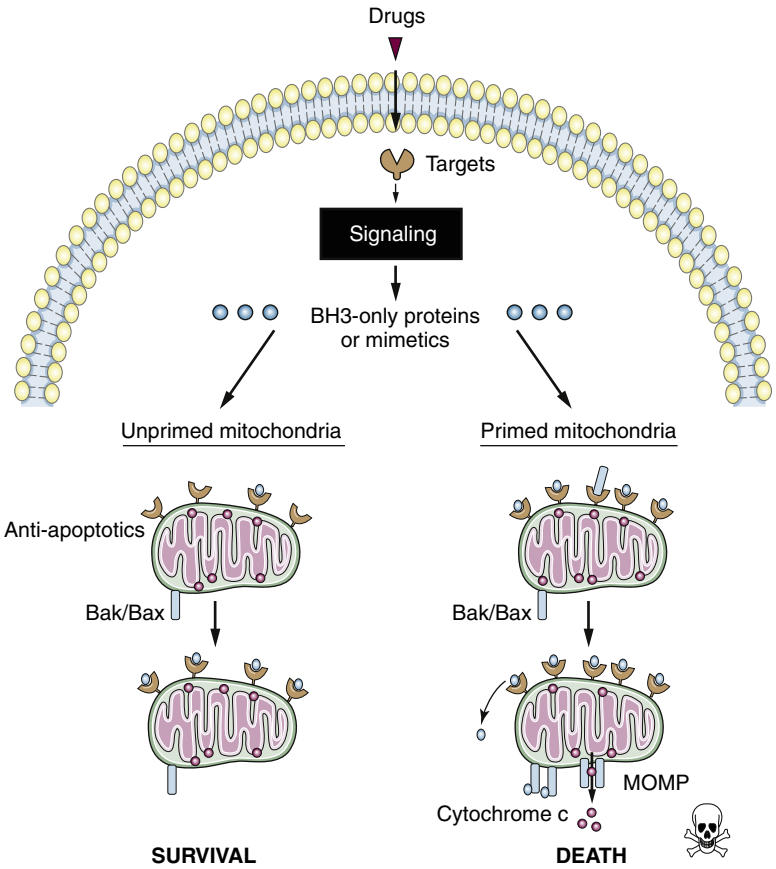


Figure 14-5 Primed versus unprimed mitochondria Functionally, primed mitochondria can be defined as those that are most sensitive to BH_3 peptides. Unprimed mitochondria *(left)* have a relative excess of "empty" anti-apoptotic proteins, affording anti-apoptotic reserve that can buffer subsequent death signaling. Primed mitochondria *(right)* may have abundant anti-apoptotic proteins, but these are relatively "full," occupied by pro-apoptotic proteins. Primed mitochondria have little anti-apoptotic reserve and are relatively sensitive to subsequent death signaling.

or in combination with others? Although some diseases may demonstrate clinical sensitivity to single agents, it may well be that combination with the powerful, albeit less selective, pro-death signaling induced by conventional cytotoxic agents will be necessary to provide the significant benefit in long-term clinical outcomes that is so badly needed in many cancers. In addition, combinations with targeted agents such as kinase inhibitors have proved promising in vitro.

The Death Receptor Pathway of Apoptosis

The death receptors are a subset of cell surface receptors that are members of the tumor necrosis factor (TNF) receptor (TNFR) superfamily. These receptors can be activated by ligands, which are themselves a subset of the TNF superfamily. The death receptors include one of the receptors for TNF, TNFR1, the TRAIL receptors, and CD95 (also called Fas or APO1).

When a death receptor is engaged by its ligand, this can result in apoptosis. In this death receptor pathway of apoptosis, the initiator caspase is caspase-8, and its activation platform involves the adapter protein, FADD, which binds to the prodomain of the caspase, allowing it to form its active dimer.

The simplest form of this process is seen in the ligation of CD95 (this may apply to the TRAIL receptors as well). Ligation of CD95 causes the exposure of a protein interaction region in the intracellular part of the receptor, which rapidly binds to FADD. Several receptors and the associated FADD form the caspase activation platform, and caspase-8 is recruited to FADD for its activation. The proteolytic activity of the bound caspase-8 now cuts the caspase, removing the prodomain and releasing the now-stabilized dimer (Figure 14-6, *A*).

In the case of TNFR1, the process is much more complex. Binding of ligand to TNFR1 induces exposure of the intracellular region of the receptor, but this does not bind FADD. Instead, another adapter, called TRADD, is bound, as well as a number of other signaling proteins, some of which modify the associated proteins with ubiquitin. Among these is RIPK1, a protein kinase, discussed further later. Also among these are proteins involved in the activation of a transcription factor, nuclear factor-κB (NFκB). The initial complex (complex I) is then released from the receptor, and its subsequent signaling outcome depends on additional protein interactions. Among these is a protein called c-$FLIP_L$ (also called CFLAR).

c-$FLIP_L$ closely resembles caspase-8 but lacks a catalytic cysteine. When complex I is released, TRADD binds to FADD in the cytosol, and this recruits caspase-8. However,

if c-FLIP$_L$ is present, the complex that forms (complex IIa) contains heterodimers of caspase-8 and c-FLIP$_L$, and this does not promote apoptosis (discussed later). However, if the activity of NFκB is blocked or disrupted, or if the expression of c-FLIP$_L$ is otherwise blocked, complex IIa activates caspase-8 (see Figure 14-6, *B*). Another complex that forms in cells exposed to TNF involves the kinase RIPK1 (complex IIb). Like TRADD, RIPK1 can bind to FADD and form a caspase-activation platform. Again, if c-FLIP$_L$ is present, caspase-8–c-FLIP$_L$ heterodimers form, and apoptosis does not proceed. However, if c-FLIP$_L$ is absent, this can also result in caspase-8 activation (see Figure 14-6, *C*).

The formation of the RIPK1-FADD complex is not restricted to the death receptor pathway. It can form as a consequence of DNA damage, although the precise signaling mechanisms are not fully elucidated.[64] Another way in which this forms is as a result of ligation of some Toll-like receptors (TLRs), molecules that recognize components of infectious organisms, such as bacteria. Some TLRs recruit a signaling molecule, called TRIF, and this can engage RIPK1 to form a complex with FADD.[65] As with the other cases, the subsequent activation of caspase-8 to promote apoptosis depends on the presence of c-FLIP$_L$. Efforts to therapeutically activate death receptor pathways for cancer therapy are in development.[66]

Mitochondrial Pathway Activation by Caspase-8

When we discussed caspase activation, we noted that initiator caspases, such as caspase-8, can cleave and thereby activate executioner caspases to promote apoptosis. However, in most cell types, the activated executioner caspases are bound by an inhibitor, X-linked inhibitor of apoptosis protein (XIAP), which then ubiquitinates the caspase and promotes its degradation. This prevents apoptosis from proceeding. However, caspase-8 has another substrate in the cell that indirectly overcomes this inhibition. This protein is BID, one of the BH3-only proteins discussed in the last section. When caspase-8 cleaves BID, this BH3-only protein translocates to the mitochondria to promote MOMP.

Although MOMP and the release of cytochrome c can then lead to activation of caspase-9, as we have noted previously, this event is not necessary in this case. Instead, the release of other proteins, such as Smac and Omi, is required, as these bind to and neutralize XIAP. As a result, the executioner caspases cleaved by caspase-8 can now precipitate apoptosis in the cell. This is illustrated in Figure 14-6, *D*. This explains the otherwise apparently paradoxical observation that anti-apoptotic Bcl-2 proteins can often inhibit apoptosis engaged by the death receptor pathway.

Necrosis

Passive and Active Necrosis

Necrosis (type III cell death) is distinct from apoptosis and autophagic cell death in terms of both morphology and mechanisms. Cells that die by necrosis generally have diffuse nuclei and a loss of organellar structures, as entry of water into the cell causes swelling and often rupture of membrane compartments.

Understanding necrosis is important in cancer biology for several reasons. Most importantly, cancers that contain high levels of necrosis often have a poor prognosis. This may be because such cancers grow so quickly that they outstrip their blood supply. Another important aspect of necrosis is that it is inflammatory: That is, the contents of the dying cells release mediators (DAMPs, see earlier discussion) that trigger inflammatory responses. Inflammation itself damages tissues and can cause cycles of repair and proliferation involving growth factors that can promote oncogenesis and tumor expansion. In many adult cancers, inflammation may be a major component of the process that promotes tumorigenesis.

As we noted earlier, it is useful to distinguish between cell death that is passive versus active, and this applies to necrosis. Passive necrosis occurs when cells are irreparably damaged by external forces, or when processes that are essential for sustaining cellular homeostasis are blocked or disrupted. This can occur as a consequence of mechanical stress or toxic chemicals or as a result of specialized pore-forming peptides or proteins (e.g., some venoms and bacterial toxins, complement, perforin). Because such processes are intuitively fairly obvious, our focus here is on forms of necrosis that are active, that is, depend on cellular processes and events that, if inhibited, preserve the survival of the cell. For the most part, these all correspond to forms of cellular "sabotage," although as we will see, there is one form of necrosis that might be "suicide."

Necrosis as an Adjunct to Other Cell Death Modalities

Before embarking on a survey of the mechanisms of active necrosis, we must note that there are processes that can confuse the distinctions between necrosis and other forms of cell death. These are secondary necrosis and caspase-independent cell death, both related to apoptosis, and many forms of autophagic cell death.

As we mentioned, when a cell dies by apoptosis, it is rapidly engulfed by other cells and removed from the system before any loss of plasma membrane integrity. However, if such engulfment does not occur, the dying cells will take on some features of necrosis as plasma membrane integrity is

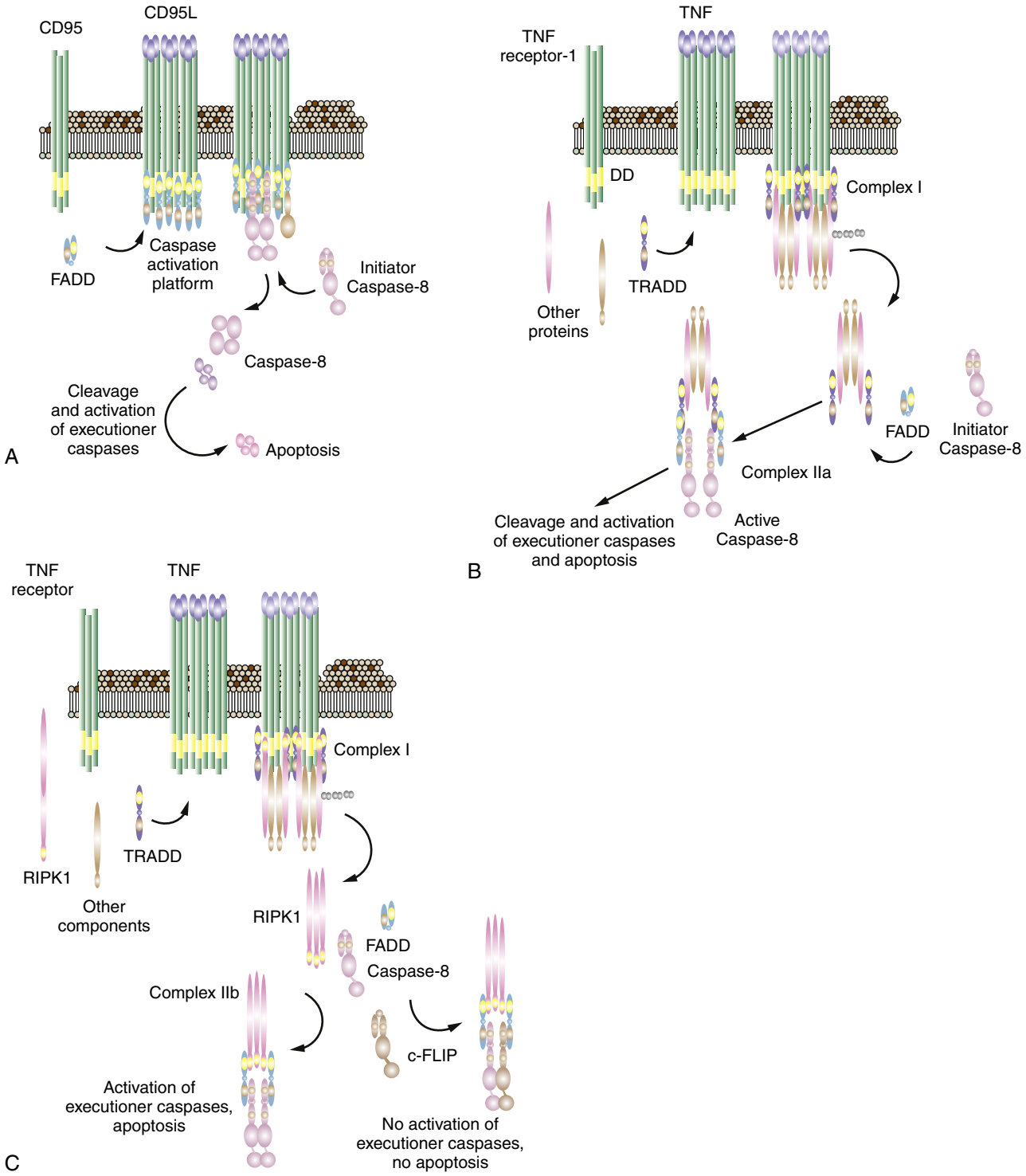


Figure 14-6 Signaling of cell death by death receptors Induction of cell death by **(A)** CD95; **(B)** TNF through complex IIa; **(C)** TNF through complex IIb; and **(D)** death receptor signaling through mitochondria. The often-used "Fas" to designate CD95 stands for "FS-7 associated surface" antigen, recognized by an antibody capable of inducing apoptosis, originally discovered by S. Yonehara. Because "FAS" designates fatty acid synthase, an unrelated protein, the term has been replaced by CD95. Unfortunately, however, the old nomenclature persists and may cause confusion.

Continued

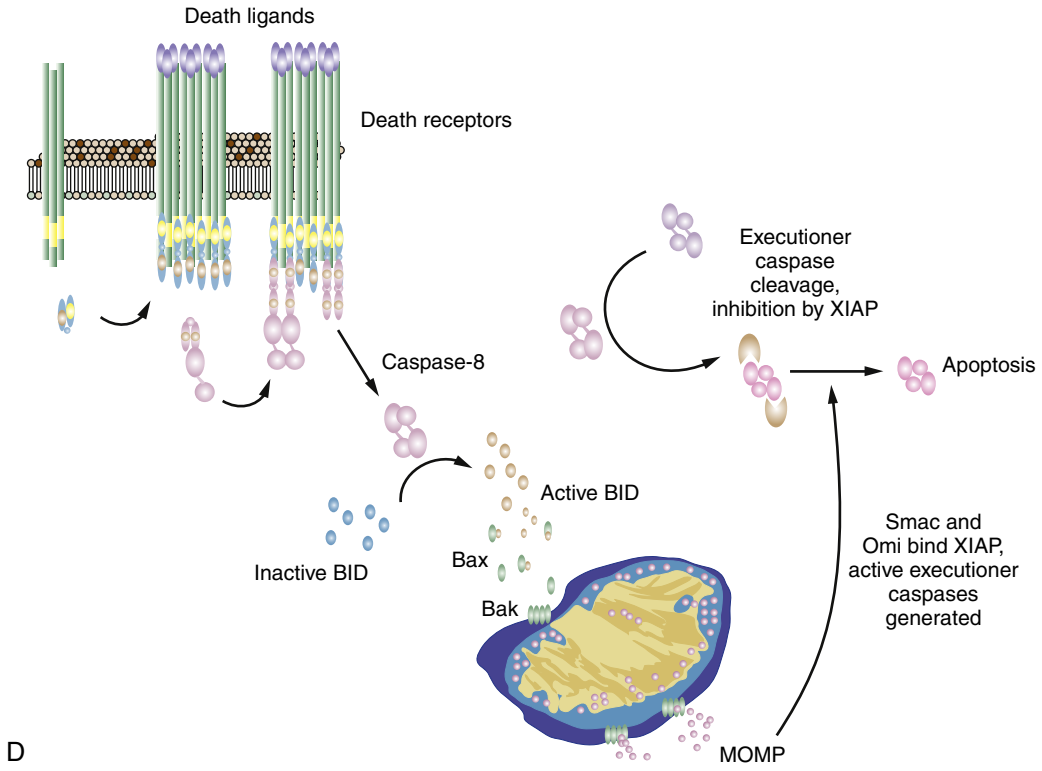


Figure 14-6, Cont'd

lost. This is called *secondary necrosis* and can often be distinguished from other forms of necrosis by some features of apoptosis, such as chromatin condensation. Whether or not secondary necrosis is inflammatory, and thus may contribute to some disease states, remains controversial, but there is accumulating evidence that defects in the engulfment and clearance of apoptotic cells can cause pathology arising from the effects of secondary necrosis.

A second form of necrosis that is related to apoptosis is *caspase-independent cell death* (CICD).[67] This occurs when a cell undergoes MOMP in the mitochondrial pathway of apoptosis, but caspase activation is blocked or disrupted. Although several mechanisms have been suggested for why CICD occurs, an emerging consensus suggests that this is mainly due to energetic consequences of the "mitochondrial catastrophe" produced by MOMP.[68] Notably, regulatory events that control MOMP, such as expression of anti-apoptotic BCL-2 proteins, effectively block CICD and thus distinguish this form of necrosis. Although cells that undergo MOMP but do not engage caspases often die by CICD, cells can survive this event,[69,70] and it is possible that such survival contributes to cancer and/or chemotherapeutic resistance in some cases.

As mentioned earlier, passive necrosis occurs when essential cellular processes are disrupted, but repair mechanisms can insulate a cell from such insults. One major repair mechanism is autophagy (discussed in much more detail later), and therefore a cell that dies as a consequence of such damage may show extensive evidence of autophagy. As a result, we may categorize cells dying in this way as so-called autophagic (type II) cell death. In such cases, however, inhibition or disruption of autophagy will render the cell more susceptible to necrosis, and therefore it may be more appropriate to regard this process as one of necrosis.

Ischemia and Necrosis

Probably the most common cause of necrosis in cancer is as a result of ischemic injury, the loss of blood supply to the tumor. There are two major ways ischemia causes necrosis; the first is via the deprivation of oxygen and nutrients. This occurs in tumors as regions of the mass are deprived of blood supply and can be regarded as passive necrosis. A second, more complex way occurs when blood supply is restricted and then regained, a condition referred to as *ischemia/reperfusion injury*. Paradoxically, reperfusion of a deprived tissue often results in massive necrosis, and this is a major clinical problem.

Ischemia/reperfusion injury is a complex process we can regard as a form of cell sabotage and thus active cell death, although it is not fully understood. There are three components of ischemia/reperfusion injury that are generally agreed on: a rise in intracellular calcium, the production of reactive oxygen species, and disruption of the mitochondria.

On reperfusion, potassium channels in the plasma membrane open, releasing potassium from the cytosol, and this in turn causes the opening of plasma membrane calcium channels and a rise in intracellular calcium (Ca^{2+}). The increase in Ca^{2+} has several effects. First, it can activate the protease calpain, which can inflict proteolytic damage to the cell. Pharmacologic inhibitors of calpain have some protective effects in ischemia/reperfusion injury. Second, Ca^{2+} activates NADPH-oxidases, causing the production of ROS, which can also damage the cell by targeting lipids and DNA. Damage to the DNA elicits activation of polyADP-ribose polymerase (PARP), and extensive activation of PARP can deplete stores of NADH, accelerating death. Scavenging ROS or inhibition of PARP can also produce some protection against this form of injury.

Both Ca^{2+} and ROS may precipitate necrosis in large part by affecting the mitochondria through a process called the *mitochondrial permeability transition*. The inner mitochondrial membrane is critical for mitochondrial function, as the control of the distribution of protons and other ions across this membrane drives ATP production and other activities of the organelle. High levels of Ca^{2+} and/or ROS cause the opening of a "pore" in the membrane that dissipates these critical gradients, and the matrix swells, ultimately destroying the organelle. The components of the permeability transition pore are largely unknown, with one exception: a protein of the mitochondrial matrix, cyclophilin D, has a major role in the process. Animals lacking cyclophilin D display no developmental abnormalities, but are resistant to ischemia/reperfusion injury. Whatever the function of the mitochondrial permeability transition might be, it may be safe to conclude that necrosis induced by ischemia/reperfusion that engages this mechanism represents a form of cellular sabotage. Whether this process can be used to promote cancer cell death therapeutically is not known.

Necroptosis Is a Form of Active Necrosis

In the discussion of the death receptor pathway of apoptosis, a kinase, RIPK1, was introduced, which participates in one of the TNFR complexes and can also function in complexes induced by other means (DNA damage, TLR-TRIF signaling). RIPK1 can also promote a form of necrosis, called *necroptosis* (to distinguish it from other forms, such as those discussed earlier).

Necroptosis involves the activation of another kinase, RIPK3, which binds to RIPK1 but can also be engaged by other proteins (a putative sensor of viral DNA, called DAI-1, activates RIPK3 and causes necroptosis independently of RIPK1). The activation of RIPK3 rapidly induces cell death with the features of necrosis.

How cell death occurs on activation of RIPK3 is largely unknown and is an area of intense research. One other player in the process is another protein, MLKL, which may itself be a kinase or may act as an adapter to bring specific substrates to RIPK3. Although a number of effector mechanisms have been proposed, including mitochondrial ROS and other mediators, at this point we simply do not know how this form of necrosis occurs.

As discussed in the consideration of death receptor signaling, RIPK1 can recruit the adapter, FADD, into a caspase activation platform that engages caspase-8 and the caspase-like molecule, c-$FLIP_L$. Although the caspase-8–c-$FLIP_L$ heterodimer is an enzymatically active protease, it does not promote apoptosis (for reasons that we still do not understand). However, it has now become clear that this activity serves an important function in cells: It blocks the activation of RIPK3 (Figure 14-7).

Ligation of TNFR1, or other ways in which RIPK1 is engaged and activated, generally does not cause necroptosis, unless the formation of the FADD–caspase-8–c-$FLIP_L$ complex is blocked or disrupted.[71,72] Mice in which the genes for FADD or caspase-8 are deleted die early in embryogenesis, but development is fully rescued by ablation of RIPK3. The effect of ablating c-FLIP is the same, but its rescue is more complex: In this case death occurs only if both RIPK3 and FADD are deleted, most likely because activity of FADD to activate caspase-8 promotes death in cells lacking c-FLIP.

How, exactly, the caspase-8–c-$FLIP_L$ heterodimer blocks necroptosis is not fully known. One simple possibility is that it cuts RIPK1 (and perhaps RIPK3), rendering it inactive. However, other possibilities exist, either upstream of RIPK1 or downstream of RIPK3. For example, an enzyme, CYLD, that removes ubiquitin from RIPK1 (required for its necroptotic function) is targeted by caspase-8[73] and therefore may also play a role in the protective function of caspase-8–c-$FLIP_L$.

Why is the system "built" this way? One possibility is that many viruses have evolved mechanisms to inhibit the activation of caspase-8 as a way to avoid immune attack, and therefore, linking RIPK3-induced necroptosis to the pathway ensures that cells infected by such viruses still die. Another, similar argument relates to c-$FLIP_L$, which is rapidly turned over in cells: When intracellular parasites (including viruses) disrupt the translation of cellular proteins, levels of c-$FLIP_L$ decline, thereby sensitizing the cell to necroptosis. The destruction of the cell not only limits the replication of the parasite, but also engages the inflammatory response elicited by the necrotic cells.

Because necroptosis appears to be a bona fide form of cellular suicide, it is possible that this cell death process can function in tumor suppression. As more is learned about this phenomenon, cancers may be found in which oncogenesis is offset by engagement of necroptosis. Alternatively, there may

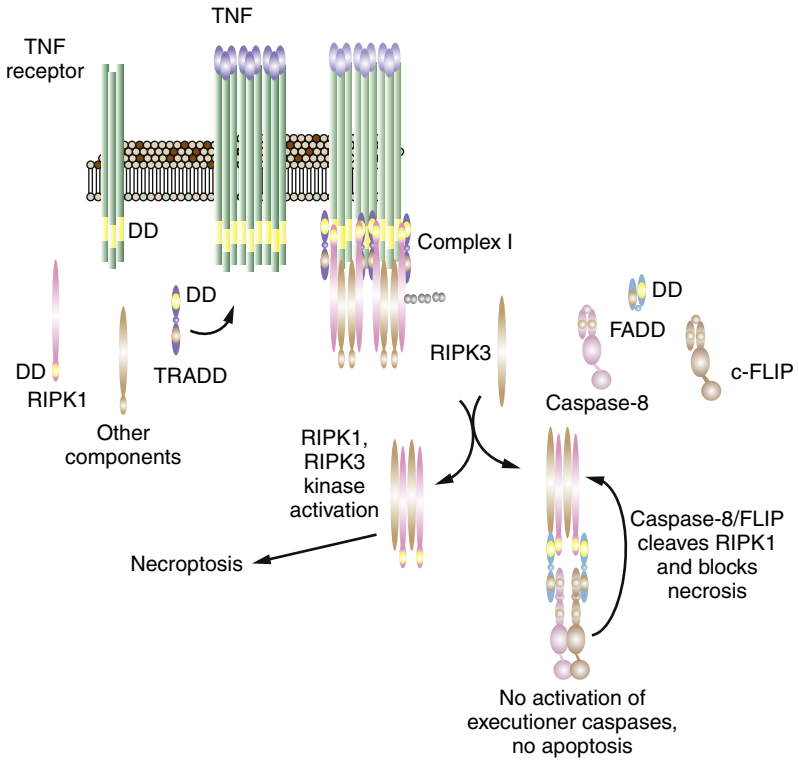


Figure 14-7 The necroptosis pathway See text for explanation.

be ways to exploit the process for tumor cell destruction in some forms of cancers.

Necroptosis, RIP Kinases, and Cancer Therapy

One area of cancer therapy involves a class of compounds called *Smac mimetics*, as they were originally designed to mimic the effects of the IAP antagonists (such as Smac) released upon MOMP. In fact, we now know that these agents work through targeting the cellular IAP molecules, c-IAP1 and c-IAP2.

The c-IAP molecules are E3 ligases that have, among their targets, the kinases RIPK1 and RIPK3. In healthy cells, the c-IAPs restrict the accumulation of these kinases and therefore prevent their activation. When cells are exposed to the Smac mimetics, the kinases accumulate, but if c-FLIP$_L$ is present, the action of FADD-caspase-8–c-FLIP$_L$ prevents death. However, if c-FLIP$_L$ is absent, the cells will die by caspase-8–mediated apoptosis or via necroptosis.

Autophagy

Autophagy, or cellular self-eating, is a process cells use to collect intracellular proteins, cytoplasm and organelles, and deliver them to lysosomes where they are degraded and recycled.[74] There are multiple forms of autophagy that use different routes to deliver cargo to lysosomes for degradation.[74] We focus here on macroautophagy (autophagy hereafter), where double membrane vesicles called *autophagosomes* capture cargo and then fuse with lysosomes. Autophagy has a dual role in cancer: It can be tumor suppressive by preserving cellular and tissue health and can be tumor promoting by supporting mitochondrial metabolism and survival in stress. We discuss the contexts in which autophagy influences cancer development, progression, and response to therapy.

Autophagy was discovered more than 50 years ago and was found to be activated by starvation in yeast, which enabled identification and characterization of many of what would become known as the approximately 30 autophagy-related (*atg*) genes.[75,76] This helped establish the framework for autophagy regulation, autophagosome formation, and the capture, delivery, and degradation of cargo in lysosomes. Discovery of orthologs of *atg* genes in other model organisms and in humans broadened our understanding of the role of autophagy in health and in disease.

There are two major functions of autophagy. The first is to eliminate damaged or superfluous proteins and organelles, commonly referred to as the protein and organelle quality control function of autophagy. The second is to degrade

and recycle intracellular components to sustain metabolism and homeostasis in the absence of external nutrients, commonly referred to as the catabolic function of autophagy. Autophagy occurs at a low basal level in normal cells and tissues and is dramatically upregulated by stress and starvation, which is critical for cellular and mammalian survival and homeostasis.[77] Variations of autophagy can also remodel tissues in development or capture and degrade intracellular pathogens and contribute to host defense.[74] Autophagy has been paradoxically referred to as type II programmed cell death, where instead autophagy is predominantly a survival mechanism. The long-ago observations of autophagosomes in dying cells that led to this designation are instead likely a result of cells attempting to save themselves by activating autophagy.[78]

Protein and Organelle Quality Control

A common feature of cells and tissues with defective autophagy is the accumulation of aggregated proteins and dysfunctional mitochondria and other organelles, which can be toxic.[79,80] Autophagy is specifically critical to purge bad mitochondria on a regular basis; otherwise cells fill up with these defective organelles. Cells can degrade individual soluble proteins via the ubiquitin-proteasome pathway; however, autophagy is the only mechanism cells have for large-scale bulk degradation and for degrading large structures such as protein aggregates and entire organelles. Autophagy is thereby a nonredundant process critical for cellular physiology. Tissues from mice deficient for essential autophagy (*atg*) genes accumulate autophagy substrates including lipids, aggregated proteins, and abnormal organelles, particularly mitochondria. Autophagy deficiency causes neurodegeneration,[81,82] liver damage and inflammation,[83-86] muscle cell deterioration,[87] degeneration of pancreatic islets,[88,89] and impairment of lymphocyte homeostasis.[90] Liver and brain appear to require autophagy more than other tissues.

Cell Catabolism

A common function of autophagy that is conserved from yeast to mammals is to provide survival in starvation through intracellular recycling.[91-94] This is dramatically demonstrated by the observation that mice deficient for the essential autophagy gene *atg5* are born but fail to survive the neonatal starvation period, and their tissues display signs of energy impairment.[92] In the absence of external nutrients, catabolic recycling of proteins and organelles by autophagy is essential to sustain mammalian metabolism and survival.

Quality Control and Catabolism Overlap

Mitochondria are the nexus of functional overlap between the quality control and catabolic functions of autophagy. On the one hand, the quality control activity of autophagy is required to preserve the functioning pool of mitochondria to sustain their metabolic activity and cell survival. On the other hand, the catabolic activity of autophagy supplies substrates to mitochondria for them to metabolize. Thus, a major role for autophagy is in the regulation of mitochondrial metabolism, which affects cellular energy status, oxidative stress, signaling, and anabolism.

Dual and Context-Specific Role for Autophagy in Cancer

Both the quality control and catabolic functions of autophagy promote cell, tissue, and organismal homeostasis and survival, and in most circumstances this suppresses or delays cell death mechanisms of apoptosis and necrosis by promoting cellular health.[95,96] Autophagy has a dual and context-specific role in cancer.[97,98] By preserving cell and tissue health in some circumstances, autophagy can be tumor suppressive. Autophagy, however, can enable survival of cancer cells (and normal cells) in stress and in response to activation of some oncogenic pathways, and in this context autophagy can be tumor promoting.

Process of Autophagy

The central functions of autophagy have at their core the ability to identify and capture cargo and deliver it to the lysosomal compartment where it is degraded.[74] The breakdown products of intracellular proteins and organelles are then released from lysosomes into the cytoplasm, where they can be reutilized.[93] These amino acids, nucleosides, sugars, and fatty acids are used either as substrates to drive metabolic pathways or as building blocks for generation of new biomass. Regulators of autophagy ensure that the right cargo is degraded at the right time in the right cells.

In normal conditions, autophagy functions at low basal levels to ensure the occasional, selective, and necessary turnover of proteins and organelles due to aging and normal wear and tear. Damaged proteins and organelles display the "eat me" signal, the addition of polyubiquitin chains to proteins, which are recognized by autophagy receptors that recruit autophagosomes. In response to stress and starvation, autophagy is robustly induced. This ensures the elimination of damaged proteins and organelles that are induced by stressful conditions and also provides an alternate source

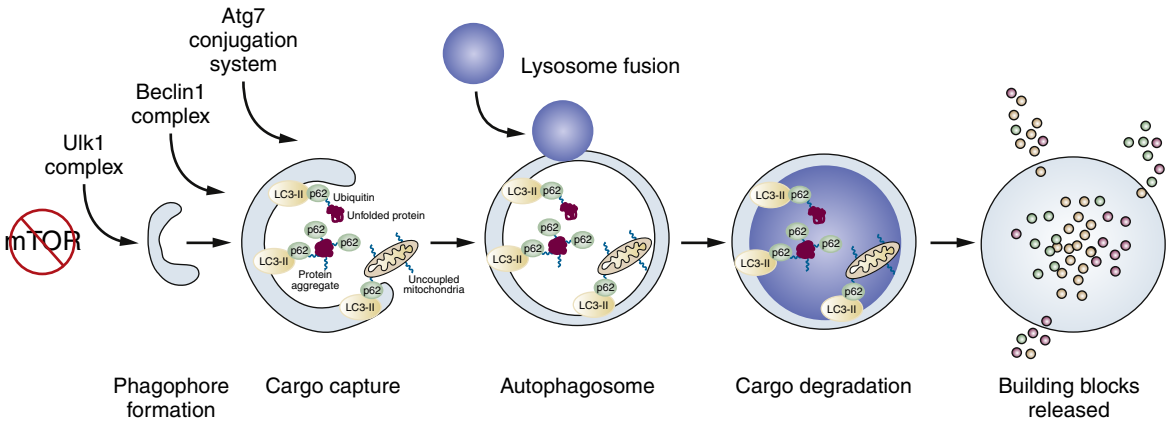


Figure 14-8 Process of autophagy See text for explanation.

of bulk metabolic substrates through intracellular recycling when external nutrient sources are limiting.

There are many growth regulatory and stress pathways that control the switch from basal to induced autophagy. Many of these pathways converge on the major nutrient-sensing and cell growth–promoting mammalian target of rapamycin (mTOR). Nutrients activate mTOR, which shuts off initiation of autophagosome formation by directly phosphorylating components of the *atg1*/unc-51–like kinase 1 (ULK1) complex (see Figure 14-8).[99] Starvation suppresses mTOR activity, de-repressing autophagy and providing a direct link between nutrient availability, cell growth, and catabolism (Figure 14-8). Simply put, cells have no need to eat themselves when there is plenty of food, and regulation of autophagy by mTOR integrates that important relationship.

mTOR is not the only way to regulate autophagy. For instance, cells regulate autophagy induction by monitoring their energy status. AMP-activated protein kinase (AMPK) is activated by interaction with AMP that accumulates when cells are energy deprived. In turn, AMPK potently activates autophagy by directly phosphorylating and activating the ULK1 complex to initiate autophagosome formation.[100,101] Stress also activates autophagy. For instance, low oxygen induces hypoxia-inducible transcription factors that turn on the expression of *atg* genes to stimulate autophagy.[102] Ammonia, a waste product of amino acid and nucleic acid metabolism, induces autophagy, linking autophagy to metabolic stress.[103,104] Activation of cancer pathways such as those controlled by oncogenic Ras,[105,106] p53 loss,[107] and activation of NFκB[108] activate autophagy, and the functional consequences are under active investigation.

The ULK1 complex activates autophagy by associating with membranes, often those of the endoplasmic reticulum, and recruiting the class III phosphatidylinositol (PtdIns) 3-kinase Vps34, Beclin1/Atg6 complex, and Atg9-containing vesicles. PtdIns production recruits double FYVE-containing protein 1 (DFCP1), which initiates autophagosome formation. Two ubiquitin-like conjugation systems governed by Atg7 form the Atg12-5-16L1 complex and process LC3/Atg8 to the cleaved, lipidated, and mature autophagosome-associated form LC3-II. The Atg5-12-16L1 complex and LC3-II function to elongate and close the autophagosome membrane. Autophagy receptors such as p62 bind cargo by interacting with their ubiquitin modifications and bind autophagosomes by interacting with LC3-II, thereby capturing the cargo in the forming autophagosome (see Figure 14-8). Autophagy can cause nonselective bulk degradation or can be highly selective, as is the case for mitophagy, which specifically targets depolarized mitochondria to the autophagy pathway for degradation.[80]

Autophagy-Mediated Tumor Suppression

The protein and organelle quality control function of autophagy plays an important role in maintaining tissue health that suppresses cancer. There are sweeping deleterious consequences to this failure to take out the "cellular garbage" that can ultimately render some tissues tumor prone. The mechanism of tumor suppression by autophagy is linked to degradation of oncogenic proteins, suppression of cell death, inflammation and chronic tissue damage, and ultimately to prevention of mutations and genetic instability (Figure 14-9).[97]

The essential autophagy gene *beclin1* was identified as a potential tumor suppressor gene when it was found to be monoallelically lost in a small cohort of human breast, ovarian, and prostate cancers and when its overexpression reduced the tumorigenicity of human cancer cell lines.[109,110] Knockout of *beclin1* in mice causes embryonic lethality; however, mice heterozygous for *beclin1* are prone to hepatocellular carcinoma, lung tumors, lymphomas, and mammary hyperplasia.[111,112] This suggested that *beclin1* is a haploinsufficient tumor suppressor. However, because it also functions in vesicle trafficking, it is not clear if this is due to its essential role in autophagy.

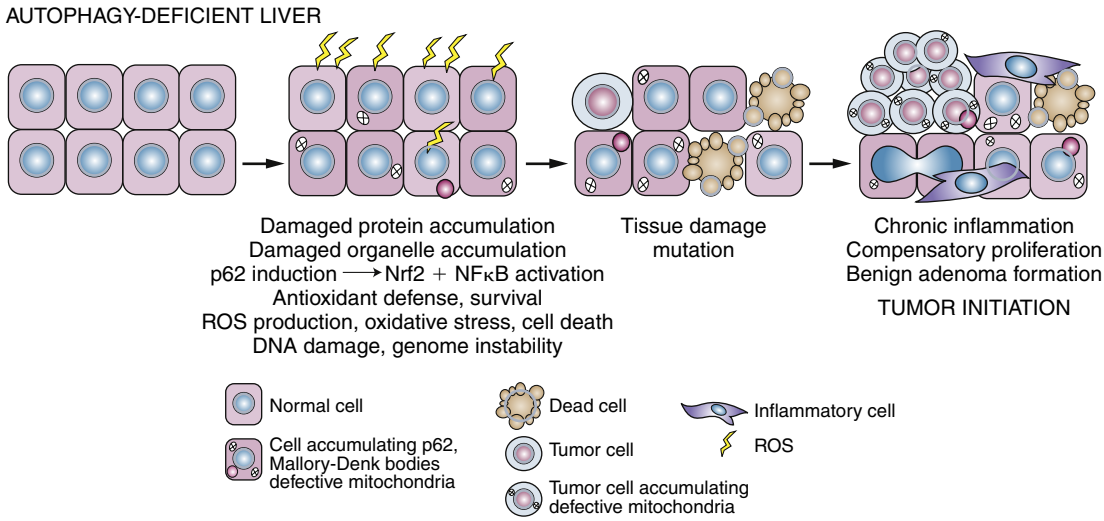


Figure 14-9 Tumor suppression by autophagy See text for explanation.

Mosaic knockout of the essential autophagy gene *atg5* in approximately 30% of cells throughout the mouse causes benign hepatoma (liver tumor) development without progression to hepatocellular carcinoma, and with no tumors in other tissues.[86] This suggests that autophagy deficiency promotes the initiation of cancer specifically in the liver, but because the neoplasms remain benign, autophagy may be required for progression to more aggressive cancers. It also suggests that tissues other than liver are less susceptible to tumor promotion caused by autophagy deficiency. Perhaps loss of other non–autophagy-related functions of *beclin1* are involved, or alternatively, partial autophagy deficiency due to allelic loss of *beclin1* may promote tumor initiation.

How autophagy defects promote cancer initiation in the liver is gradually emerging. Deficiency in *atg5* or *atg7* or allelic loss of *beclin1* in the liver causes fatty liver disease, and accumulation of abnormal mitochondria, the autophagy substrate p62, and p62- and ubiquitin-modified protein aggregates.[84-86] These p62 aggregates are also known as *Mallory-Denk bodies*, which are a hallmark of human liver disease and hepatocellular carcinoma. How these multiple aspects of autophagy deficiency may predispose to liver tumor promotion is then the question.

It is clear that degradation of the autophagy substrate p62 contributes to tumor suppression in the liver. p62 is a signaling adaptor protein that regulates cancer-promoting pathways, for instance, that of Nrf2 and NFκB.[113] This raised the possibility that deregulated levels of p62 resulting from impaired autophagy could promote the activation of pro-oncogenic pathways. p62 binds Keap1, the negative regulator of Nrf2, resulting in Nrf2 activation and upregulation of transcription of antioxidant defense genes that promote survival to oxidative stress.[83,114] Loss of autophagy and the resulting p62 accumulation activates Nrf2. Concurrent deficiency in *atg7* and *p62* prevents liver tumor initiation and enforced p62 accumulation in autophagy-deficient cancer cell lines accelerates tumorigenesis.[84-86,115] These findings demonstrate that p62 accumulation that results from autophagy defects promotes tumor initiation in the liver and can accelerate tumor progression.

p62 also interacts with TRAF6 and promotes NFκB activation. Autophagy defects and aberrant p62 accumulation and altered NFκB regulation may also contribute to the development of liver tumors.[85] Accumulation of p62 in aggregates blocks NFκB activation in hepatocytes, promoting cell death and triggering chronic inflammation that is known to cause liver cancer.[85,116] Suppression of inflammation may be another important tumor suppression mechanism mediated by autophagy independent of Nrf2 inhibition. In support of this concept, partial loss of *atg16L1*, which has been linked to the cancer-prone inflammatory condition Crohn disease, or deficiency in *atg5*, promotes Paneth cell dysfunction and inflammation of intestinal epithelium.[117]

Cancer development and progression can be facilitated by upregulation of Nrf2 and inflammation but ultimately requires acquisition of genetic changes. Autophagy defects activate the DNA damage response, suggesting that chronic tissue damage and ROS production can potentially generate cancer-causing genome mutations.[86,118-120] Indeed, autophagy-deficient tumor cells display an increased mutation rate, chromosome copy number variations, and genome instability compared to autophagy-functional tumor cells.[118,120] Thus, suppression of genetic instability by autophagy is likely the ultimate mechanism by which tumor suppression occurs. These and other findings have collectively suggested that autophagy stimulation may be beneficial for cancer prevention and the mechanism behind the health benefits of caloric restriction, fasting, and exercise that promote autophagy.[97]

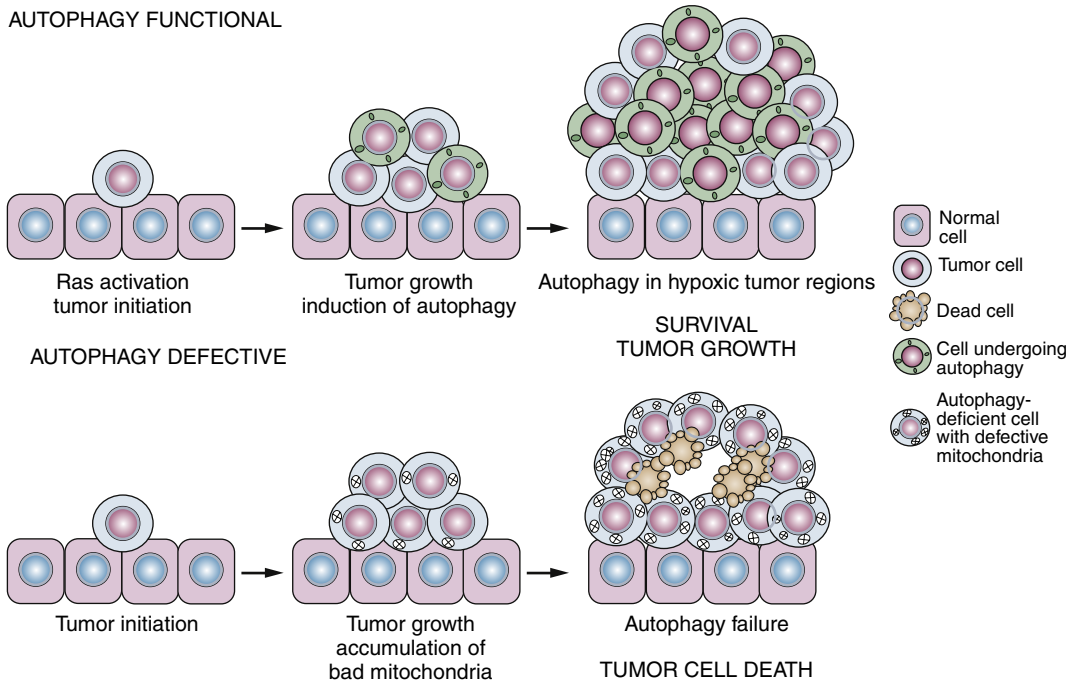


Figure 14-10 Tumor promotion by autophagy See text for explanation.

Tumor Promotion by Autophagy

Normal cells, tissues, and mammals require autophagy to sustain metabolism in starvation. Tumor cells also require autophagy for survival, but because tumor cells often reside in a metabolically stressed environment, their requirement for autophagy is often increased compared to normal cells. For instance, autophagy is upregulated in, and required for survival of, tumor cells that reside in hypoxic tumor regions, typically in the tumor center.[96,118,120] This is why tumor cells deficient for autophagy have a pronounced survival disadvantage in response to metabolic stress in vitro and form hollow tumors in comparison to autophagy-proficient tumor cells (Figure 14-10). Stressed, autophagy-deficient tumor cells die by necrosis or apoptosis, indicating that metabolic insufficiency is incompatible with survival and is independent of a specific cell death mechanism.

Activation of oncogenic pathways rewires metabolism to promote cell growth and elevate metabolic demand.[121] This suggests that tumor cells may have an intrinsic requirement for elevated autophagy to help meet this increased metabolic demand. Indeed, high basal autophagy is a common characteristic of human and mouse cancer cell lines bearing activated oncogenic *H-ras* or *K-ras* oncogenes.[105,106,122] These include human pancreatic cancer cell lines and tumors that have a high prevalence of activating *K-ras* mutations.[106] Moreover, introduction of activated Ras that confers tumorigenicity is sufficient to cause high basal autophagy.[105] Whether other oncogenic events also upregulate basal autophagy is not known; however, high levels of autophagosomes correlating with aggressive disease have been reported in some human tumors.[123]

High basal autophagy in Ras-driven cancers is required for survival in stress and for tumorigenesis. For instance, mouse cancer cell lines with activated Ras are highly tumorigenic, whereas those deficient for *atg* genes are defective for tumor growth.[105] Stable knockdown of essential autophagy genes in human cancer cell lines also compromises growth and survival in vitro and impairs tumor growth in vivo.[105,106] Human pancreatic cancer cell line xenographs were also highly sensitive to autophagy inhibition with hydroxychloroquine (HCQ),[106] which interferes with lysosome function and blocks the degradation of autophagy cargo delivered to the lysosomal compartment for degradation.[124] These findings suggest that cancer cells can be addicted to autophagy to survive stress and sustain tumor growth. This has stimulated interest in inhibiting autophagy in established tumors to improve cancer therapy.

Analysis of cancer cells and tumors with and without autophagy is beginning to reveal the possible mechanism of autophagy addiction. In contrast to tumor cell lines with functional autophagy, those that are autophagy deficient accumulate defective mitochondria.[105,106] The mitochondria from autophagy-deficient tumor cells are morphologically abnormal and display aberrant ROS production, reduced respiration, and depletion of key tricarboxylic acid (TCA) cycle metabolites in starvation.[105,106] Thus, tumor cells need autophagy to maintain the functional pool of mitochondria required for efficient tumor growth. Although tumors

upregulate glycolytic metabolism, they still require mitochondria for anabolic purposes such as citrate production for lipid and new membrane synthesis and to produce signaling levels of ROS essential for tumor growth.[125,126]

Role of Autophagy in Cancer Therapy

The survival-promoting function of autophagy in stressed tumor cells and the autophagy addiction of Ras-driven cancers support the concept that autophagy inhibition may be a useful approach to enhance cancer therapy.[97,98,127] Moreover, most anticancer therapeutics induce autophagy, either indirectly because they are cytotoxic, or directly because they block pathways that inhibit autophagy, such as those upstream of mTOR, and even mTOR itself.[128] This has suggested that therapeutic autophagy induction may be a resistance mechanism and that combination with autophagy inhibition may enhance efficacy.[97,98,127] Clinical trials are under way to test this hypothesis using HCQ, more potent analogs of HCQ are being assessed,[129] and new autophagy inhibitors targeting different points in the autophagy pathway are in development. Combining autophagy inhibition with nutritional stress[130] or targeted metabolic inhibitors is also promising.

Key References

1. Green DR, Evan GI. A matter of life and death. *Cancer Cell*. 2002;1:19-30.
2. Kroemer G, Galluzzi L, Vandenabeele P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*. 2009;16:3-11.
3. Galluzzi L, Vitale I, Abrams JM, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ*. 2012;19:107-120.
4. Ryoo HD, Baehrecke EH. Distinct death mechanisms in *Drosophila* development. *Curr Opin Cell Biol*. 2010;22:889-895.
5. Green DR, Victor B. The pantheon of the fallen: why are there so many forms of cell death? *Trends Cell Biol*. 2012;22:555-556.
6. Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. *J Biol Chem*. 2009;284:21777-21781.
7. Green DR. *Means to an End: Apoptosis and Other Cell Death Mechanisms*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2011.
9. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004;116:205-219.
11. Kroemer G, Jaattela M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer*. 2005;5:886-897.
12. Tait SW, Green DR. Caspase-independent cell death: leaving the set without the final cut. *Oncogene*. 2008;27:6452-6461.
13. Martin SJ, Henry CM, Cullen SP. A perspective on mammalian caspases as positive and negative regulators of inflammation. *Mol Cell*. 2012;46:387-397.
17. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci*. 2009;122:437-441.
18. Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer*. 2008;8:121-132.
19. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev *Mol Cell*. Biol 2010;11:621-632.
23. Willis SN, Fletcher JI, Kaufmann T, et al. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*. 2007;315:856-859.
24. Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351-365.
27. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*. 2005;17:393-403.
28. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*. 2005;17:525-535.
29. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003;426:671-676.
30. Davids MS, Letai A. Targeting the B-cell lymphoma/leukemia 2 family in cancer. *J Clin Oncol*. 2012;30(25):3127-3135.
32. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature*. 2004;432:307-315.
33. Biswas S, Shi Q, Matise L, Cleveland S, Dave U, Zinkel S. A role for proapoptotic Bax and Bak in T-cell differentiation and transformation. *Blood*. 2010;116:5237-5246.
36. Wertz IE, Kusam S, Lam C, et al. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature*. 2011;471:110-114.
37. Tan TT, Degenhardt K, Nelson DA, et al. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell*. 2005;7:227-238.
38. Kutuk O, Letai A. Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells. *Cell Death Differ*. 2010;17:1624-1635.
39. Brunelle JK, Ryan J, Yecies D, Opferman JT, Letai A. MCL-1-dependent leukemia cells are more sensitive to chemotherapy than BCL-2-dependent counterparts. *J Cell Biol*. 2009;187:429-442.
44. Chipuk JE, Kuwana T, Bouchier-Hayes L, et al. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*. 2004;303:1010-1014.
45. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell*. 2003;11:577-590.
46. Ni Chonghaile T, Sarosiek KA, Vo TT, et al. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science*. 2011;334:1129-1133.
47. Vo T-T, Ryan J, Carrasco R, et al. Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell*. 2012;151:344-355.
48. Moore VDG, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest*. 2007;117:112-121.
49. Deng J, Carlson N, Takeyama K. Dal Cin P, Shipp M, Letai A. BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell*. 2007;12:171-185.
50. Roberts AW, Seymour JF, Brown JR, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol*. 2012;30:488-496.
51. Al-Harbi S, Hill BT, Mazumder S, et al. An antiapoptotic BCL-2 family expression index predicts the response of chronic lymphocytic leukemia to ABT-737. *Blood*. 2011;118:3579-3590.
53. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
54. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
55. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004;101:13306-13311.

56. Kuroda J, Puthalakath H, Cragg MS, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci U S A*. 2006;103:14907-14912.
58. Gong Y, Somwar R, Politi K, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med*. 2007;4:e294.
59. Deng J, Shimamura T, Perera S, et al. Proapoptotic BH3-only BCL-2 family protein BIM connects death signaling from epidermal growth factor receptor inhibition to the mitochondrion. *Cancer Res*. 2007;67:11867-11875.
60. Takezawa K, Okamoto I, Nishio K, Janne PA, Nakagawa K. Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer. *Clin Cancer Res*. 2011;17:2140-2148.
61. Shao Y, Aplin AE. Akt3-mediated resistance to apoptosis in B-RAF-targeted melanoma cells. *Cancer Res*. 2010;70:6670-6681.
62. Ng KP, Hillmer AM, Chuah CT, et al. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. *Nat Med*. 2012;18:521-528.
63. Qin JZ, Ziffra J, Stennett L, et al. Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res*. 2005;65:6282-6293.
64. Tenev T, Bianchi K, Darding M, et al. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell*. 2011;43:432-448.
65. Feoktistova M, Geserick P, Kellert B, et al. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell*. 2011;43:449-463.
66. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene*. 2013;32:1341-1350.
67. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol*. 2005;6:268-275.
68. Lartigue L, Kushnareva Y, Seong Y, Lin H, Faustin B, Newmeyer DD. Caspase-independent mitochondrial cell death results from loss of respiration, not cytotoxic protein release. *Mol Biol Cell*. 2009;20:4871-4884.
69. Colell A, Ricci JE, Tait S, et al. GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell*. 2007;129:983-997.
70. Tait SW, Parsons MJ, Llambi F, et al. Resistance to caspase-independent cell death requires persistence of intact mitochondria. *Dev Cell*. 2010;18:802-813.
71. Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. *Mol Cell*. 2011;44:9-16.
72. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature*. 2011;471:363-367.
73. O'Donnell MA, Perez-Jimenez E, Oberst A, et al. Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat Cell Biol*. 2011;13:1437-1442.
74. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011;147:728-741.
75. Klionsky DJ, Cregg JM, Dunn Jr WA, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell*. 2003;5:539-545.
76. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol*. 2009;10:458-467.
77. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell*. 2004;15:1101-1111.
78. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol*. 2008;9:1004-1010.
79. Lamark T, Johansen T. Aggrephagy: selective disposal of protein aggregates by macroautophagy. *Int J Cell Biol*. 2012 2012:736905.
80. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*. 2010;12:9-14.
81. Komatsu M, Waguri S, Chiba T, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 2006;441:880-884.
82. Hara T, Nakamura K, Matsui M, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 2006;441:885-889.
83. Komatsu M, Kurokawa H, Waguri S, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12:213-223.
84. Komatsu M, Waguri S, Koike M, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell*. 2007;131:1149-1163.
85. Mathew R, Karp CM, Beaudoin B, et al. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. 2009;137:1062-1075.
86. Takamura A, Komatsu M, Hara T, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev*. 2011;25:795-800.
87. Wu JJ, Quijano C, Chen E, et al. Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging (Albany, NY)*. 2009;1:425-437.
88. Jung HS, Chung KW, Won Kim J, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab*. 2008;8:318-324.
89. Ebato C, Uchida T, Arakawa M, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab*. 2008;8:325-332.
90. Pua HH, He YW. Autophagy and lymphocyte homeostasis. *Curr Top Microbiol Immunol*. 2009;335:85-105.
91. Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol*. 2005;169:425-434.
92. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432:1032-1036.
93. Rabinowitz JD, White E. Autophagy and metabolism. *Science*. 2010;330:1344-1348.
94. Suzuki SW, Onodera J, Ohsumi Y. Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction. *PLoS One*. 2011;6:e17412.
95. Boya P, Gonzalez-Polo RA, Casares N, et al. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol*. 2005;25:1025-1040.
96. Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006;10:51-64.
97. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer*. 2012;12:401-410.
98. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res*. 2009;15:5308-5316.
99. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
100. Egan DF, Shackelford DB, Mihaylova MM, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science*. 2011;331:456-461.
101. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol*. 2011;13:1016-1023.
102. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev*. 2010;20:51-56.
103. Cheong H, Lindsten T, Wu J, Lu C, Thompson CB. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. *Proc Natl Acad Sci U S A*. 2011;108:11121-11126.
104. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. *Sci Signal*. 2010; 3(119):ra31.
105. Guo JY, Chen HY, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. 2011;25:460-470.
106. Yang S, Wang X, Contino G, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011;25:717-729.
107. Tasdemir E, Maiuri MC, Galluzzi L, et al. Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol*. 2008;10:676-687.
108. Criollo A, Senovilla L, Authier H, et al. IKK connects autophagy to major stress pathways. *Autophagy*. 2010;6:189-191.
111. Qu X, Yu J, Bhagat G, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*. 2003;112:1809-1820.

112. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A*. 2003;100:15077-15082.
113. Moscat J, Diaz-Meco MT. p62: a versatile multitasker takes on cancer. *Trends Biochem Sci*. 2012;37:230-236.
114. Lau A, Wang XJ, Zhao F, et al. A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol Cell Biol*. 2010;30:3275-3285.
115. Inami Y, Waguri S, Sakamoto A, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J Cell Biol*. 2011;193:275-284.
116. Sun B, Karin M. NF-kappaB signaling, liver disease and hepatoprotective agents. *Oncogene*. 2008;27:6228-6244.
117. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature*. 2008;456:259-263.
118. Karantza-Wadsworth V, Patel S, Kravchuk O, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev*. 2007;21:1621-1635.
119. Lee IH, Kawai Y, Fergusson MM, et al. Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress. *Science*. 2012;336:225-228.
120. Mathew R, Kongara S, Beaudoin B, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007;21:1367-1381.
121. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. 2012;21:297-308.
122. Lock R, Roy S, Kenific CM, et al. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. *Mol Biol Cell*. 2010;22:165-178.
123. Ma XH, Piao S, Wang D, et al. Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin Cancer Res*. 2011;17:3478-3489.
124. Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest*. 2007;117:326-336.
125. Mullen AR, Wheaton WW, Jin ES, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*. 2011;481:385-388.
126. Weinberg F, Hamanaka R, Wheaton WW, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci U S A*. 2010;107:8788-8793.
127. Amaravadi RK, Lippincott-Schwartz J, Yin XM, et al. Principles and current strategies for targeting autophagy for cancer treatment. *Clin Cancer Res*. 2011;17:654-666.
128. Bray K, Mathew R, Lau A, et al. Autophagy suppresses RIP kinase-dependent necrosis enabling survival to mTOR inhibition. *PLoS One*. 2012;7:e41831.
129. McAfee Q, Zhang Z, Samanta A, et al. Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. *Proc Natl Acad Sci U S A*. 2012;109:8253-8258.
130. Sheen JH, Zoncu R, Kim D, Sabatini DM. Defective regulation of autophagy upon leucine deprivation reveals a targetable liability of human melanoma cells in vitro and in vivo. *Cancer Cell*. 2011;19:613-628.

This page intentionally left blank

References

1. Green DR, Evan GI. A matter of life and death. *Cancer Cell*. 2002;1:19-30.
2. Kroemer G, Galluzzi L, Vandenabeele P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*. 2009;16:3-11.
3. Galluzzi L, Vitale I, Abrams JM, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ*. 2012;19:107-120.
4. Ryoo HD, Baehrecke EH. Distinct death mechanisms in *Drosophila* development. *Curr Opin Cell Biol*. 2010;22:889-895.
5. Green DR, Victor B. The pantheon of the fallen: why are there so many forms of cell death? *Trends Cell Biol*. 2012;22:555-556.
6. Pop C, Salvesen GS. Human caspases: activation, specificity, and regulation. *J Biol Chem*. 2009;284:21777-21781.
7. Green DR. *Means to an End: Apoptosis and Other Cell Death Mechanisms*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2011.
8. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972;26:239-257.
9. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell*. 2004;116:205-219.
10. Wolf BB, Green DR. Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem*. 1999;274:20049-20052.
11. Kroemer G, Jaattela M. Lysosomes and autophagy in cell death control. *Nat Rev Cancer*. 2005;5:886-897.
12. Tait SW, Green DR. Caspase-independent cell death: leaving the set without the final cut. *Oncogene*. 2008;27:6452-6461.
13. Martin SJ, Henry CM, Cullen SP. A perspective on mammalian caspases as positive and negative regulators of inflammation. *Mol Cell*. 2012;46:387-397.
14. Bakhshi A, Jensen JP, Goldman P, et al. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell*. 1985;41:899-906.
15. Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci U S A*. 1985;82:7439-7443.
16. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science*. 1985;228:1440-1443.
17. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci*. 2009;122:437-441.
18. Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer*. 2008;8:121-132.
19. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev *Mol Cell*. Biol 2010;11:621-632.
20. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*. 2001;292:727-730.
21. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*. 2002;2:183-192.
22. Wei MC, Lindsten T, Mootha VK, et al. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev*. 2000;14:2060-2071.
23. Willis SN, Fletcher JI, Kaufmann T, et al. Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*. 2007;315:856-859.
24. Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9:351-365.
25. Cheng EH, Wei MC, Weiler S, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*. 2001;8:705-711.
26. Chittenden T, Flemington C, Houghton AB, et al. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J*. 1995;14:5589-5596.
27. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*. 2005;17:393-403.
28. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*. 2005;17:525-535.
29. Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*. 2003;426:671-676.
30. Davids MS, Letai A. Targeting the B-cell lymphoma/leukemia 2 family in cancer. *J Clin Oncol*. 2012;30(25):3127-3135.
31. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
32. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature*. 2004;432:307-315.
33. Biswas S, Shi Q, Matise L, Cleveland S, Dave U, Zinkel S. A role for pro-apoptotic Bax and Bak in T-cell differentiation and transformation. *Blood*. 2010;116:5237-5246.
34. McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature*. 1991;349:254-256.
35. Symonds H, Krall L, Remington L, et al. p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell*. 1994;78:703-711.
36. Wertz IE, Kusam S, Lam C, et al. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature*. 2011;471:110-114.
37. Tan TT, Degenhardt K, Nelson DA, et al. Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell*. 2005;7:227-238.
38. Kutuk O, Letai A. Displacement of Bim by Bmf and Puma rather than increase in Bim level mediates paclitaxel-induced apoptosis in breast cancer cells. *Cell Death Differ*. 2010;17:1624-1635.
39. Brunelle JK, Ryan J, Yecies D, Opferman JT, Letai A. MCL-1-dependent leukemia cells are more sensitive to chemotherapy than BCL-2-dependent counterparts. *J Cell Biol*. 2009;187:429-442.
40. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*. 1993;74:957-967.
41. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*. 2001;7:683-694.
42. Oda E, Ohki R, Murasawa H, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*. 2000;288:1053-1058.
43. Zhan Q, Fan S, Bae I, et al. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene*. 1994;9:3743-3751.
44. Chipuk JE, Kuwana T, Bouchier-Hayes L, et al. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*. 2004;303:1010-1014.
45. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell*. 2003;11:577-590.
46. Ni Chonghaile T, Sarosiek KA, Vo TT, et al. Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science*. 2011;334:1129-1133.
47. Vo T-T, Ryan J, Carrasco R, et al. Relative mitochondrial priming of myeloblasts and normal HSCs determines chemotherapeutic success in AML. *Cell*. 2012;151:344-355.
48. Moore VDG, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest*. 2007;117:112-121.
49. Deng J, Carlson N, Takeyama K. Dal Cin P, Shipp M, Letai A. BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell*. 2007;12:171-185.
50. Roberts AW, Seymour JF, Brown JR, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol*. 2012;30:488-496.
51. Al-Harbi S, Hill BT, Mazumder S, et al. An antiapoptotic BCL-2 family expression index predicts the response of chronic lymphocytic leukemia to ABT-737. *Blood*. 2011;118:3579-3590.
52. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*. 2002;99:3530-3539.

53. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
54. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
55. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004;101:13306-13311.
56. Kuroda J, Puthalakath H, Cragg MS, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci U S A*. 2006;103:14907-14912.
57. Joensuu H, Roberts PJ, Sarlomo-Rikala M, et al. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med*. 2001;344:1052-1056.
58. Gong Y, Somwar R, Politi K, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med*. 2007;4:e294.
59. Deng J, Shimamura T, Perera S, et al. Proapoptotic BH3-only BCL-2 family protein BIM connects death signaling from epidermal growth factor receptor inhibition to the mitochondrion. *Cancer Res*. 2007;67:11867-11875.
60. Takezawa K, Okamoto I, Nishio K, Janne PA, Nakagawa K. Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer. *Clin Cancer Res*. 2011;17:2140-2148.
61. Shao Y, Aplin AE. Akt3-mediated resistance to apoptosis in B-RAF-targeted melanoma cells. *Cancer Res*. 2010;70:6670-6681.
62. Ng KP, Hillmer AM, Chuah CT, et al. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. *Nat Med*. 2012;18:521-528.
63. Qin JZ, Ziffra J, Stennett L, et al. Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res*. 2005;65:6282-6293.
64. Tenev T, Bianchi K, Darding M, et al. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell*. 2011;43:432-448.
65. Feoktistova M, Geserick P, Kellert B, et al. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell*. 2011;43:449-463.
66. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene*. 2013;32:1341-1350.
67. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? *Nat Rev Mol Cell Biol*. 2005;6:268-275.
68. Lartigue L, Kushnareva Y, Seong Y, Lin H, Faustin B, Newmeyer DD. Caspase-independent mitochondrial cell death results from loss of respiration, not cytotoxic protein release. *Mol Biol Cell*. 2009;20:4871-4884.
69. Colell A, Ricci JE, Tait S, et al. GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell*. 2007;129:983-997.
70. Tait SW, Parsons MJ, Llambi F, et al. Resistance to caspase-independent cell death requires persistence of intact mitochondria. *Dev Cell*. 2010;18:802-813.
71. Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. *Mol Cell*. 2011;44:9-16.
72. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature*. 2011;471:363-367.
73. O'Donnell MA, Perez-Jimenez E, Oberst A, et al. Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat Cell Biol*. 2011;13:1437-1442.
74. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011;147:728-741.
75. Klionsky DJ, Cregg JM, Dunn Jr WA, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell*. 2003;5:539-545.
76. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol*. 2009;10:458-467.
77. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell*. 2004;15:1101-1111.
78. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol*. 2008;9:1004-1010.
79. Lamark T, Johansen T. Aggrephagy: selective disposal of protein aggregates by macroautophagy. *Int J Cell Biol*. 2012 2012:736905.
80. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*. 2010;12:9-14.
81. Komatsu M, Waguri S, Chiba T, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 2006;441:880-884.
82. Hara T, Nakamura K, Matsui M, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*. 2006;441:885-889.
83. Komatsu M, Kurokawa H, Waguri S, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12:213-223.
84. Komatsu M, Waguri S, Koike M, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell*. 2007;131:1149-1163.
85. Mathew R, Karp CM, Beaudoin B, et al. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. 2009;137:1062-1075.
86. Takamura A, Komatsu M, Hara T, et al. Autophagy-deficient mice develop multiple liver tumors. *Genes Dev*. 2011;25:795-800.
87. Wu JJ, Quijano C, Chen E, et al. Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging (Albany, NY)*. 2009;1:425-437.
88. Jung HS, Chung KW, Won Kim J, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab*. 2008;8:318-324.
89. Ebato C, Uchida T, Arakawa M, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab*. 2008;8:325-332.
90. Pua HH, He YW. Autophagy and lymphocyte homeostasis. *Curr Top Microbiol Immunol*. 2009;335:85-105.
91. Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol*. 2005;169:425-434.
92. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432:1032-1036.
93. Rabinowitz JD, White E. Autophagy and metabolism. *Science*. 2010;330:1344-1348.
94. Suzuki SW, Onodera J, Ohsumi Y. Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction. *PLoS One*. 2011;6:e17412.
95. Boya P, Gonzalez-Polo RA, Casares N, et al. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol*. 2005;25:1025-1040.
96. Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell*. 2006;10:51-64.
97. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer*. 2012;12:401-410.
98. White E, DiPaola RS. The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res*. 2009;15:5308-5316.
99. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149:274-293.
100. Egan DF, Shackelford DB, Mihaylova MM, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science*. 2011;331:456-461.
101. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol*. 2011;13:1016-1023.
102. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev*. 2010;20:51-56.
103. Cheong H, Lindsten T, Wu J, Lu C, Thompson CB. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. *Proc Natl Acad Sci U S A*. 2011;108:11121-11126.
104. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. *Sci Signal*. 2010; 3(119):ra31.

105. Guo JY, Chen HY, Mathew R, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev*. 2011;25:460-470.
106. Yang S, Wang X, Contino G, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev*. 2011;25:717-729.
107. Tasdemir E, Maiuri MC, Galluzzi L, et al. Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol*. 2008;10:676-687.
108. Criollo A, Senovilla L, Authier H, et al. IKK connects autophagy to major stress pathways. *Autophagy*. 2010;6:189-191.
109. Aita VM, Liang XH, Murty VV, et al. Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics*. 1999;59:59-65.
110. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 1999;402:672-676.
111. Qu X, Yu J, Bhagat G, et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest*. 2003;112:1809-1820.
112. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A*. 2003;100:15077-15082.
113. Moscat J, Diaz-Meco MT. p62: a versatile multitasker takes on cancer. *Trends Biochem Sci*. 2012;37:230-236.
114. Lau A, Wang XJ, Zhao F, et al. A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol Cell Biol*. 2010;30:3275-3285.
115. Inami Y, Waguri S, Sakamoto A, et al. Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. *J Cell Biol*. 2011;193:275-284.
116. Sun B, Karin M. NF-kappaB signaling, liver disease and hepatoprotective agents. *Oncogene*. 2008;27:6228-6244.
117. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature*. 2008;456:259-263.
118. Karantza-Wadsworth V, Patel S, Kravchuk O, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev*. 2007;21:1621-1635.
119. Lee IH, Kawai Y, Fergusson MM, et al. Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress. *Science*. 2012;336:225-228.
120. Mathew R, Kongara S, Beaudoin B, et al. Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev*. 2007;21:1367-1381.
121. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell*. 2012;21:297-308.
122. Lock R, Roy S, Kenific CM, et al. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. *Mol Biol Cell*. 2010;22:165-178.
123. Ma XH, Piao S, Wang D, et al. Measurements of tumor cell autophagy predict invasiveness, resistance to chemotherapy, and survival in melanoma. *Clin Cancer Res*. 2011;17:3478-3489.
124. Amaravadi RK, Yu D, Lum JJ, et al. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest*. 2007;117:326-336.
125. Mullen AR, Wheaton WW, Jin ES, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*. 2011;481:385-388.
126. Weinberg F, Hamanaka R, Wheaton WW, et al. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci U S A*. 2010;107:8788-8793.
127. Amaravadi RK, Lippincott-Schwartz J, Yin XM, et al. Principles and current strategies for targeting autophagy for cancer treatment. *Clin Cancer Res*. 2011;17:654-666.
128. Bray K, Mathew R, Lau A, et al. Autophagy suppresses RIP kinase-dependent necrosis enabling survival to mTOR inhibition*PLoS One*. 2012;7 e41831.
129. McAfee Q, Zhang Z, Samanta A, et al. Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. *Proc Natl Acad Sci U S A*. 2012;109:8253-8258.
130. Sheen JH, Zoncu R, Kim D, Sabatini DM. Defective regulation of autophagy upon leucine deprivation reveals a targetable liability of human melanoma cells in vitro and in vivo. *Cancer Cell*. 2011;19:613-628.

Jessica E. Bolden and Scott W. Lowe

15 Cellular Senescence

The term *senescence* was coined more than 50 years ago to describe the loss of replicative capacity of normal human diploid cells in culture.[1] At that time, senescence was proposed to generally reflect the process of cellular aging.[2,3] Early studies also noted differences between the propensity of normal and malignant cells to senesce, with malignant cells being frequently "immortalized" or capable of unlimited subcultivation in vitro. These observations were some of the first conceptual links between the bypass of replicative senescence and tumorigenesis.

Cellular senescence is now appreciated to be much more than a passive cell-autonomous antiproliferative program reflecting the normal aging process. In fact, senescence is a key cellular program that can be induced and plays an important role in permanently restricting the propagation of damaged and defective cells. Hence, senescence responses can be induced prematurely in actively dividing cells both in vitro and in vivo through the application of endogenous and exogenous stimuli that are associated with proliferative stress and/or evoke DNA damage. Such stimuli include the aberrant expression and/or activation of oncogenes, direct DNA damage caused by exposure to ionizing radiation, reactive oxygen species, and chemotherapeutic drugs. Consequently, the integrity of the senescence program can have an active impact on cellular stress responses, cancer development and treatment outcome.

Although the process of cellular senescence is not a precise cellular counterpart of normal organismal aging, genes important for the execution of senescence have been linked to longevity, and it is clear that senescent cells accumulate in tissues with age. Interestingly, beyond the cell-autonomous control of the cell cycle arrest program, senescent cells actively secrete molecules that influence the behavior of neighboring cells, resulting in the paracrine induction of senescence, tissue remodeling, and recruitment of immune cells. Thus senescent cells may contribute to the etiology of age-related disease by restricting the proliferation of neighboring stem-cell populations needed for ongoing tissue rejuvenation, and through its secretory program modulate tissue remodeling and inflammation.

Still, the most exciting recent developments in the senescence field relate to the biology of cancer, with ramifications for understanding of the tumorigenic process, therapy responses, and new approaches to treat the disease. This chapter outlines the molecular basis of senescence and highlights the importance of this permanent cytostatic program as a protective mechanism against the propagation of damaged/defective cells. We discuss the physiological roles of senescence in vivo, focusing on its contributions as a barrier to cellular transformation.

Biochemical and Morphological Characteristics of Senescent Cells

To date, most studies describing the senescence phenotype have been performed in fibroblasts, although at least some senescence characteristics occur in epithelial and hematopoietic tissues as well. Despite induction of replicative and premature senescence by diverse stimuli, the biochemical and morphological characteristics of senescent cells are similar (Figure 15-1). In vitro, senescent cells can be identified on the basis of their large flattened morphology, a lack of DNA replication (detected by the reduced incorporation of 5-bromodeoxyuridine or ^{3}H-thymidine), increased expression of proteins associated with cell cycle arrest and tumor suppression (such as the tumor suppressor p53 and cyclin-dependent kinase inhibitors [CDKi] $p16^{INK4A}$, $p21^{CIP1/WAF1}$, and $p15^{INK4B}$), and the presence of senescence-associated β-galactosidase activity, which is attributed to the high lysosomal content of senescent cells. Senescent cells undergo marked changes in chromatin structure, as characterized by the presence of densely staining senescence-associated heterochromatic foci (SAHF), and also display altered histone modification profiles. Finally, senescent cells secrete a diverse array of proinflammatory and extracellular matrix

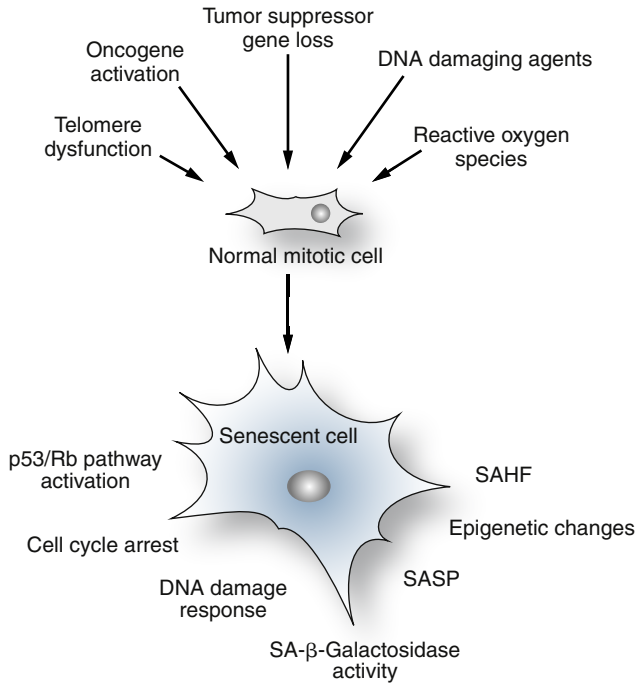


Figure 15-1 Senescence stimuli and biological characteristics Senescence is induced by diverse endogenous and exogenous stresses that promote strong pro-proliferative signaling and DNA damage responses. Senescent cells undergo a permanent proliferative arrest and are identified by the combined presence of multiple biochemical and morphological characteristics. In vitro senescent cells display an enlarged and flattened morphology, have elevated senescence-associated β-galactosidase activity, and express markers consistent with RB and p53 tumor suppressor pathway activation, cell cycle arrest, and DNA damage response signaling. Senescent cells also undergo marked changes in chromatin organization (the formation of senescence-associated heterochromatic foci—SAHF), undergo epigenetic changes, and secrete a diverse collection of soluble and insoluble factors (the senescence-associated secretory phenotype—SASP).

remodeling factors, collectively referred to as the *senescence-associated secretory phenotype* (SASP).

The challenges associated with studying senescence in vivo have been a roadblock in the advancement of the senescence field. In contrast to apoptosis, another program that restricts proliferation and tumorigenesis, the morphological changes associated with senescence are difficult to visualize in whole tissues, and there are no simple assays (analogous to assessing DNA fragmentation or caspase activation during apoptosis) for definitively identifying senescent cells histologically. Thus senescent cells are predominantly identified in vivo by the presence of a collection of biochemical marks. Unfortunately, many "senescence markers" are not unique to the program; for example, upregulation of certain CDKi also occurs in quiescent cells. Moreover, the combination of biochemical marks expressed by given senescent cells can be cell-type and stimulus dependent. Despite these limitations, cells with combinations of senescent markers have been observed in aged, damaged and fibrotic tissues, in premalignant lesions, and in malignant tumors following chemotherapy, suggesting key processes in which the program might participate.

Replicative Senescence and the Hayflick Limit

Studies by Hayflick and colleagues[1-3] demonstrated that most normal human diploid cells could not be subcultured beyond about 50 passages in vitro—the so-called Hayflick limit. On reaching this limit, cells remained irreversibly arrested with a senescent morphology, even when presented with growth factors in an optimal proliferative environment. Although later reports demonstrated that the cellular lifespan of clones within a bulk population is more variable than originally proposed,[3] the original concept that normal cells invariably stop dividing in culture, even in optimal growth conditions, holds true.

Over the ensuing decades, the Hayflick limit was shown to occur as a consequence of accumulated telomere erosion and dysfunction.[4] Telomeres are complex structures consisting of repetitive DNA sequences ($[TTAGGG]_n$ in vertebrates) and specialized proteins that form protective caps on the ends of linear chromosomes to prevent their recognition as a DNA break.[5] The "directional" nature of DNA replication prevents the replication of the extreme ends of telomeres (the "end replication problem"); thus telomeric DNA shortens with every cell division.[5] With repeated divisions, telomeres can become critically short and fail to form the protective cap, resulting in activation of DNA damage signaling and the onset of replicative senescence[6-8] and thereby preventing cellular immortalization. Even when senescence is prevented through a variety of genetic perturbations in the program, ongoing telomere dysfunction creates a state of chromosomal instability called *crisis* that limits proliferation.[9]

In order for cancer cells to bypass senescence and become immortal, they must acquire an ability to regulate telomere length and/or integrity. The addition of telomeric DNA repeats can be catalyzed by the enzyme telomerase.[10] On rare occasions cells can aberrantly upregulate the expression of telomerase (or elongate telomeres through alternative pathways), enabling bypass of replicative senescence and crisis. This effectively facilitates the unlimited propagation of cells with chromosomal fusions and genomic instability, a critical step preceding cellular transformation.[9] Indeed, expression of telomerase alone is sufficient to delay or completely abrogate the onset of replicative senescence in certain cell types, which provides definitive evidence linking telomere shortening to the onset of senescence.[11]

Most normal cells do not express telomerase and are therefore susceptible to replicative senescence. However, telomerase is expressed by normal cells that are dependent on long-term proliferative potential for their biological function, such as germ, stem, and progenitor cells.[9] Although the expression of telomerase alone is insufficient to transform human cells,[12] telomerase activity is often associated with human cell immortalization and is upregulated in many cancer cells.[13] Moreover, expression of telomerase is a key factor in an oncogene "cocktail" capable of fully transforming normal human fibroblasts.[14] Hence, strategies to target telomerase for cancer control have received much attention.

Senescence and Viral Oncoproteins

The study of oncoproteins encoded by DNA tumor viruses has been instrumental in enabling the study of senescence and its contribution to cellular immortalization and transformation. Specifically, the expression of oncoproteins such as SV40 large T antigen, human papillomavirus (HPV) E6 and E7 proteins, and adenoviral E1A proteins can bypass cellular senescence under appropriate conditions. Moreover, such oncoproteins can collaborate with constitutively activated Ras proteins to transform normal cells,[15-19] implying indirectly that senescence provides a barrier to malignant transformation, at least in vitro. Furthermore, sustained expression of these viral oncoproteins is required to maintain the immortalized/transformed state. For example, knockdown of HPV E6/E7 proteins is sufficient to induce senescence in HeLa and cervical cancer cell lines.[20-23] It is now appreciated that these viral oncoproteins disable the retinoblastoma (RB) and p53 proteins, which are key tumor suppressors and central regulators of the senescence program (see later discussion). Collectively these studies helped reveal that the senescence program is genetically controlled and, indeed, might play a role in limiting cancer.

Premature Senescence

Senescence is more than merely a cell division clock that regulates the proliferative lifespan of normal cells. "Premature" senescence is an active cytostatic program that is triggered in response to proliferative or genotoxic stress, such as the expression of strong oncogenes, tumor suppressor loss, exposure to DNA damage, and reactivation of tumor suppressor pathways. Unlike replicative senescence, premature senescence can be induced irrespective of the replicative "age" of cells, is independent of telomere attrition, and cannot be overridden by restoration of telomerase activity.[24]

The first example of *oncogene-induced senescence* was described in 1997, where forced expression of an oncogenic allele of Ras induced a senescence response in primary human and rodent cells that was accompanied by the induction of p53 and $p16^{INK4A}$.[25] Inactivation of p53 or $p16^{INK4A}$ was sufficient to enable the proliferation of Ras-expressing rodent cells, and co-expression of adenoviral protein E1A and Ras was sufficient to enable senescence bypass in human cells. Of note, this study implied that oncogene-induced senescence acted as an important barrier to uncontrolled proliferation during multistep tumorigenesis and also provided a biological mechanism for the observed cooperation between Ras and immortalizing oncogenes (such as viral oncoproteins) alluded to earlier.

The Ras proteins are master regulators of pathways that cooperate to drive cell proliferation, growth, and survival. Dissection of Ras signaling through the use of engineered Ras mutants and activated forms of downstream signaling components identified the Raf/MEK/MAPK cascade as the major arm of Ras signaling that triggered senescence.[26,27] Hence, activation of the very same pathways that promote cellular transformation can also drive senescence when tumor suppressor genes are intact, thus implying that senescence can serve as an antiproliferative response to aberrant mitogenic cues.

Oncogene-induced senescence is not the only form of premature senescence that has been described. In fact, many forms of cellular damage, including exposure to ionizing radiation, cytotoxic drugs, and oxidative stress, can induce a cytostatic program that is phenotypically indistinguishable from senescence. Gene expression profiling studies support the notion that these senescence programs are related and demonstrate a strong similarity to the canonical replicative senescence program triggered by telomere erosion. Cellular senescence thus appears to represent a common response to cellular stress.

Interestingly, virtually all of the known stimuli that induce senescence—including telomere malfunction and hyperproliferation—can activate a DNA damage response (DDR), suggesting that some aspects of DDR signaling are crucial triggers of senescence. Accordingly, abrogation of DNA damage signaling through mutation/deletion of key regulators such as ATM, NBS1, CHK2, and ATR suppresses senescence, largely due to a failure to activate the p53 pathway.[7,28] Furthermore, DDR signaling can trigger generation of the SASP[29] and the global chromatin changes that are observed in senescent cells.[30]

Generally, it appears that a substantial damage "threshold" must be reached before replicative or premature senescence responses can be triggered. For example, cells with minor DNA damage may arrest only transiently, providing an opportunity for the repair of genetic lesions. However,

when DNA damage is extensive, cells may opt to undergo cell death or enter senescence. This notion of dose dependence holds true for oncogene-induced senescence, which is partly governed by the induction of negative feedback mechanisms that control mitogenic signaling.[31] High levels of Ras causing hyperreplication, extreme mitogenic stress, negative feedback signaling, and a strong DDR are required for Ras-induced senescence, whereas mutation of Ras without overexpression instead promotes proliferation and cellular transformation.[32,33]

Senescence in Vivo

Traditionally, oncologists have relied on cytotoxic chemotherapy regimens for the treatment of patients with diverse tumor types. Because many of these agents cause widespread DNA damage, it is perhaps not surprising that senescence can be induced in tumor cells following treatment with chemotherapy. Early demonstrations of therapy-induced senescence in vivo employed a transgenic mouse model of lymphoma, where lymphomas engineered to be unable to apoptose underwent senescence in response to the chemotherapeutic agent cyclophosphamide, and the animals harboring these lymphomas showed prolonged survival. By contrast, mice harboring lymphomas in which senescence was also disabled responded much more poorly to chemotherapy and displayed a very poor prognosis.[34] Senescence markers are also detectable in patient tumor biopsies following neoadjuvant chemotherapy,[35,36] suggesting that therapy-induced-senescence is not just a phenomenon restricted to preclinical tumor models but an important determinant of therapeutic outcomes in patients.

Senescent cells have been identified in a variety of premalignant lesions, including lung adenomas, pancreatic intraductal neoplasias, chemically induced skin papillomas,[37] lymphocytes,[38] pituitary hyperplasia,[39] prostatic intraepithelial neoplasia,[40,41] and serrated colon hyperplasia.[42] A compelling example highlighting the potential importance of this program involves the occurrence of senescent melanocytes in benign melanocytic nevi (moles).[43] Most nevi harbor Ras pathway mutations, yet are capable of remaining in a growth-arrested state for decades.[44,45] In these nevi, melanocytes express hallmarks of senescent cells, including upregulation of $p16^{INK4A}$ and senescence-associated β-galactosidase activity.[43] Nevi are effectively poised for malignant progression following bypass of senescence (e.g., via deletion or silencing of $p16^{INK4A}$); thus, in the context of melanoma, oncogene-induced senescence genuinely protects premalignant cells from tumor progression. The fact that Ras pathway mutations and INK4a loss commonly occur in human melanomas provides genetic support for the program as a barrier to cancer.[46]

As occurs after activation of oncogenic Ras, senescence can also be triggered in vivo following the loss of tumor suppressor genes. For example, prostate-specific deletion of PTEN, a phosphatase responsible for suppressing mitogenic signals, evokes a senescence response that is dependent on p53 and opposes the development of late-stage invasive prostate tumors.[40] Irrespective of the proposed stimulus, the studies just mentioned strongly noted the conspicuous absence of senescent cells in malignant tumors, consistent with the notion that senescence acts as a barrier to tumorigenesis that must be disabled before tumors can progress. Ongoing studies of senescence continue to identify key regulators of the program that are also capable of influencing the tumorigenic process in animal models and human tumors.

Convergence of Senescence Stimuli on Two Major Pathways

The senescence-associated cell cycle arrest typically involves interplay between the RB and p53 tumor suppressor pathways (Figure 15-2), which are two of the most frequently disabled pathways in human cancer. Indeed, mutations compromising some aspect of each pathway may be a prerequisite for the formation of an advanced cancer. Crosstalk between RB and p53 occurs at multiple levels in their respective signaling hierarchies; thus the pathways actively modulate and reinforce each other to promote the senescence response. Accordingly, most human cells require defects in both pathways to efficiently bypass senescence. By contrast, inactivation of either p53 or RB is sufficient to bypass senescence in mouse embryo fibroblasts, though whether this reflects species differences in pathway wiring or merely variation between cell types is unclear.

$p16^{INK4A}$/RB Pathway

RB is a member of a multigene family that also includes the structurally and functionally related proteins p107 and p130.[47] The tumor-suppressive capacity of RB predominantly arises in part from its ability to repress the E2F family of transcription factors and thereby regulate G_1-S phase cell cycle transition. It is now appreciated that RB also controls many aspects of tumor biology, including apoptosis, differentiation, and the maintenance of chromosomal stability.[47]

RB is frequently inactivated in diverse types of human cancer, and experiments with genetically modified mice have revealed a causal role for RB loss in tumor initiation and progression in a variety of different tissue types.[47] Hints that RB might be a senescence regulator came from studies that identified associations between RB and viral oncoproteins

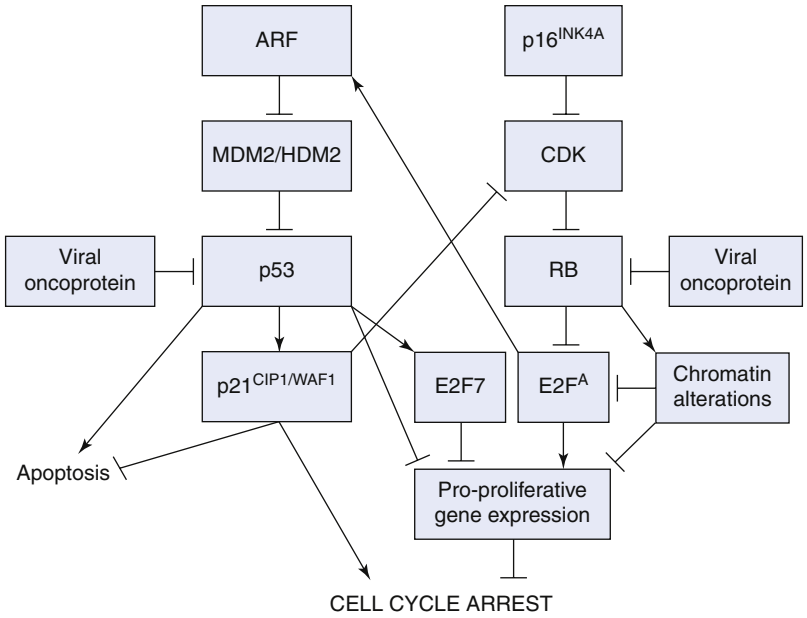


Figure 15-2 The RB and p53 tumor suppressor pathways cooperatively control the senescence cell cycle arrest In normal cells, p53 is maintained at low steady-state levels through activity of the E3 ligase MDM2 (HDM2 in humans). Hyperproliferative stresses, such as oncogene expression, can drive the induction of ARF, which sequesters MDM2, resulting in the stabilization of p53. Following DNA damage response signaling, p53 becomes hyperphosphorylated and stabilized, thus enabling its activity as a DNA-binding transcription factor. p53 transcriptionally upregulates the cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1/WAF1}$, which promotes cell cycle arrest by inhibiting cyclin/cdk2 complexes, and thereby activating RB. RB is expressed in proliferating and noncycling cells, and its activity is primarily regulated at the posttranslational level.[47] As dividing cells approach the G_1/S boundary of the cell cycle, RB is phosphorylated by cyclin D/CDK4 and cyclin E/cdk2 complexes, causing the release of activator E2F factors ($E2F^A$) from RB and transcriptional activation of E2F target genes required for DNA synthesis. During senescence, elevation of CDK inhibitors including $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ ultimately promotes hypophosphorylation of RB, inhibition of E2F-responsive gene expression, and arrest of cells at the G_1/S checkpoint. Multiple opportunities exist for crosstalk between the pathways, including the p53-mediated induction of $p21^{CIP1/WAF1}$ and E2F7 (a repressor E2F family member), which operate at different levels in the signaling hierarchy to reinforce RB pathway activity. In addition, deregulation of $E2F^A$ activity leads to upregulation of ARF, thus activating p53 signaling to initiate a secondary proliferative block.[48] Viral oncoproteins disable both p53 and RB to bypass senescence and facilitate cell immortalization. Some components of the pathways are tumor suppressors in their own right. *CDKN2A* (encoding $p16^{INK4A}$) is frequently mutated and/or lost in a variety of human tumors, and there is extensive evidence for gene silencing through methylation of the $p16^{INK4A}$ promoter in tumors.[49] $p16^{INK4A}$-null mouse embryonic fibroblasts (MEFs) possess normal growth characteristics and can senesce in response to oncogenic Ras but have increased immortalization rates in comparison with wild-type MEFs, and $p16^{INK4A}$-null mice are predisposed to tumorigenesis.[50] Consistent with ARF acting as a major regulator of p53 during oncogene-induced senescence, fibroblasts isolated from ARF-deficient embryos do not senesce and are transformed by oncogenic Ras alone.[51] ARF-deficient mice are also tumor prone.[51]

with immortalizing activity. Nevertheless, a clear-cut interpretation of these observations was confounded by the fact that these oncoproteins bind all three RB family members.[52] Moreover, RB, p107, and p130 have partly redundant functions and can often compensate for each other if one member is inactivated.[53-55] Studies using conditional knockouts[56] and RNA interference[57,58] have since revealed a unique role for RB during senescence, such that specific depletion of RB (without affecting p130 or p107) can cooperate with p53 loss to bypass senescence. The fact that RB, and not p107 and p130, is a frequently mutated tumor suppressor gene suggests that control of senescence contributes to its tumor-suppressive role.

In human cells, RB inhibits senescence by binding to E2F factors and preventing the expression of a series of E2F-responsive genes linked to DNA replication.[58] RB achieves this by inhibiting E2F proteins capable of otherwise activating growth-promoting genes and/or by recruiting chromatin-modifying factors to E2F-responsive promoters that repress gene transcription.[59] During senescence, RB also inhibits E2F-induced gene expression by incorporating E2F target genes into dense heterochromatic regions known as *SAHF*.[57] Each SAHF is thought to consist of a single condensed chromosome, enriched in histone modifications and proteins that are typically associated with transcriptionally silent heterochromatin, and largely devoid of sites of active transcription.[57,60] Consistent with these observations, proteomic analyses of the histone content of senescent cells indicate that they generally harbor modifications linked with gene repression, and certain enzymes (e.g., Jarid histone demethylases) associated with gene repression are required for the repression of some E2F target genes during senescence.[61]

The RB-directed changes just described appear to buffer E2F target genes from activation by normal mitogenic cues, thereby contributing to the stable cell cycle arrest that is a hallmark of senescence. Accordingly, disruption of RB prevents SAHF formation and gene silencing in cells given a senescence stimulus.[57] However, inactivation of RB alone is insufficient to bypass senescence in many cell types. This

is largely due to compensatory activation of the p53 pathway, which modulates an independent set of antiproliferative genes and also reinforces RB signaling to provide a secondary proliferative block.

ARF/p53/p21$^{CIP1/WAF1}$ Pathway

The ARF/p53/p21$^{CIP1/WAF1}$ tumor suppressor pathway is a major regulator of cellular responses to oncogenic stress and DNA damage, resulting in the induction of cell cycle arrest, apoptosis, and senescence, depending on the stimulus. Cells isolated from p53-deficient mice are largely resistant to stimuli that would promote growth arrest or apoptosis under normal culture conditions,[62] and p53-deficient mouse embryo fibroblasts bypass the senescence response that usually occurs following extensive DNA damage or forced expression of oncogenic Ras.[25] Not only is loss of p53 required for bypass of senescence; restoration of p53 in Ras-transformed cells causes the induction of senescence and the subsequent regression of established tumors, demonstrating that continual suppression of p53 is required for tumor maintenance.[63-65] Beyond their biological significance, these data suggest that p53-reactivation "therapies" may hold promise for the treatment of established tumors by promoting a senescence response.

p53 primarily exerts its antitumor effects by acting as a DNA-binding transcription factor that directly regulates the expression of many genes involved in apoptosis and cell cycle arrest. The effect of p53 during senescence is due, in part, to the direct transcriptional upregulation of the cyclin/cdk2 inhibitor p21$^{CIP1/WAF1}$. By inhibiting cyclin/cdk2, p21$^{CIP1/WAF1}$ promotes activation of the p16^{INK4A}/RB pathway and subsequent cell cycle arrest, thus serving as an important point of crosstalk between the two central pathways of the senescence program. In support of this hypothesis, cosuppression of p21$^{CIP1/WAF1}$ and RB pathway components (RB or p16^{INK4A}) by RNA interference inhibits senescence in human fibroblasts, highlighting p21$^{CIP1/WAF1}$ as a critical component of the p53 pathway.[58]

Deletion of p21$^{CIP1/WAF1}$ alone is not sufficient to bypass senescence in mouse embryo fibroblasts,[66] suggesting that additional p53 targets contribute to the senescence response. Such targets include the p53-responsive gene PML, which is induced by oncogenic Ras and promotes senescence by localizing RB/E2F proteins into PML bodies, thereby repressing E2F transcriptional activity.[67-69]

Another recently described point of crosstalk between the p53 and RB pathways involves the p53 transcriptional target E2F7 (an atypical E2F family member), which represses a subset of E2F target genes required for mitotic progression.[70,71] As seen for p21$^{CIP1/WAF1}$, co-inhibition of E2F7 and RB is sufficient to circumvent Ras-mediated senescence and enable transformation of mouse embryo fibroblasts.[70] Collectively, these studies highlight the interplay between the p53 and RB pathways that govern institution of the senescence proliferative block and thereby the suppression of tumorigenesis. The extent to which the control of senescence contributes to the tumor-suppressive function in human cancer remains to be determined, but the co-occurrence of Ras, p53, and RB mutations in human tumors suggests that disruption of senescence may be required for cells to tolerate high levels of oncogenic signaling and become fully malignant.

The Senescence-Associated Secretory Phenotype

Previously, we have focused largely on the cell-intrinsic mechanisms that govern senescence responses and tumorigenesis—these mechanistically explain much of the biology underlying the cell cycle arrest associated with the Hayflick limit. Senescent cells also secrete a multitude of factors, predominantly proinflammatory cytokines, chemokines, growth factors, and extracellular matrix remodeling factors, that have collectively been referred to as the senescence-associated secretory phenotype (SASP—Figure 15-3). Initially, SASP factors were used as biomarkers to confirm the presence of senescent cells. Now, emerging evidence indicates that the SASP acts in an autocrine and paracrine manner to influence the senescence program and profoundly alter the behavior of neighboring cells. This secretory program may contribute to the natural role of senescence in aged or damaged tissues.

The contribution of the SASP toward the biology of senescence and tissues is complex and can promote and suppress tumorigenesis depending on the context. Perhaps the greatest consequence of the SASP in vivo, in terms of tumor suppression, chemotherapy-induced senescence, and disease resolution, is enhanced immune surveillance and subsequent clearance of senescent cells. In such scenarios, the secretion of proinflammatory cytokines and chemokines by senescent cells attracts innate and adaptive immune cells (including natural killer cells, macrophages, and T cells) to the site of senescence and cell/tissue damage. The immune cells subsequently kill and clear the senescent cells, restoring tissue homeostasis.[63,72]

Senescent cells also upregulate the expression of cell surface receptors, ligands, and intracellular signaling components that facilitate recognition of senescent cells and aid their elimination by immune cells.[72,73] Indeed, immune surveillance of senescent cells has been positively linked to regression of established tumors,[63] the prevention of tumor

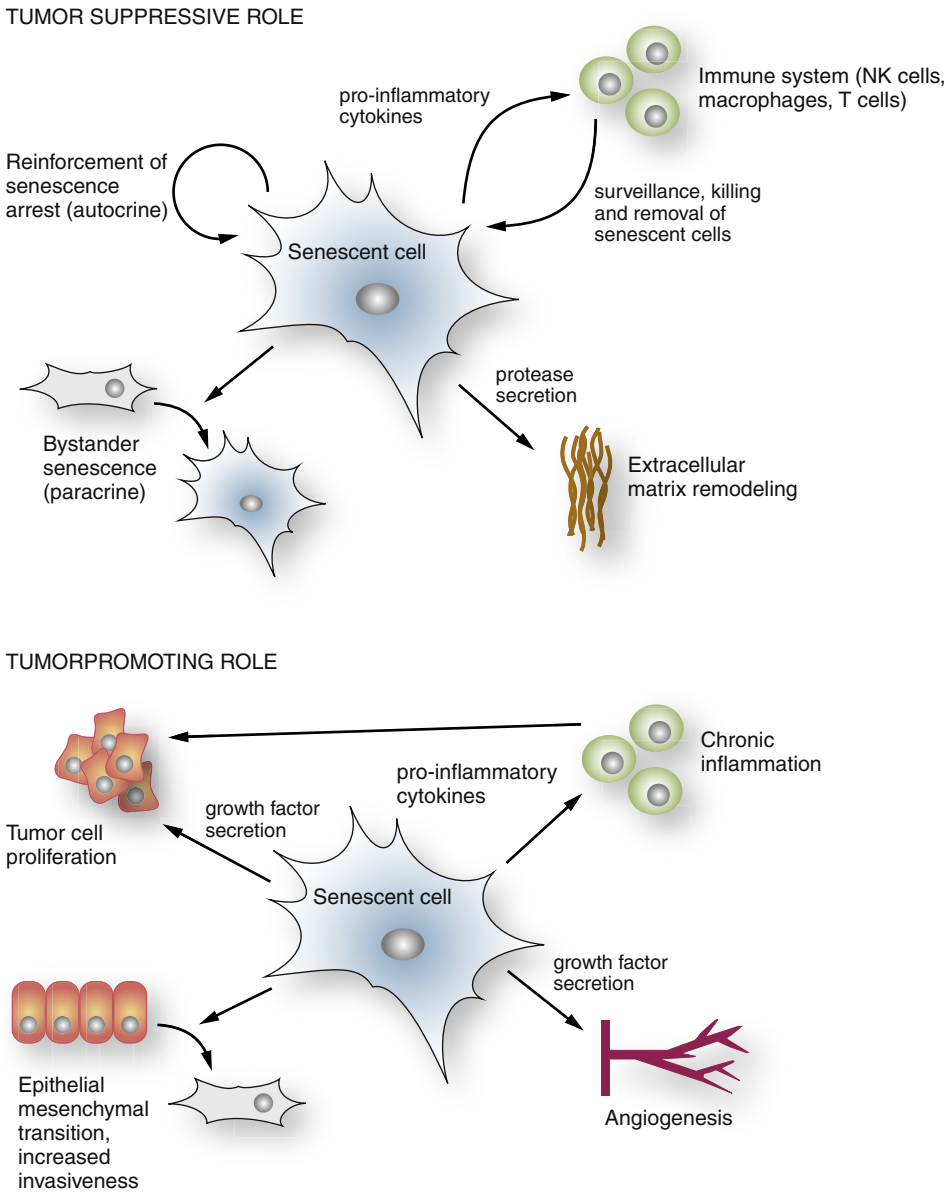


Figure 15-3 Tumor suppressive and tumor-promoting activities of the SASP Senescent cells secrete a range of factors that profoundly affect the tissue microenvironment. In the context of tumor suppression, wound healing, and disease resolution, factors secreted by senescent cells attract components of the innate and adaptive immune system, which recognize, kill, and clear the senescent cells. This results in removal of damaged/stressed cells and restoration of tissue integrity. Senescent cells secrete molecules that actively reinforce the senescence arrest and can induce secondary "bystander" senescence in neighboring cells. The secretion of proteases enables remodeling of the extracellular matrix, which facilitates resolution of tissue disease (such as fibrosis) and thereby promotes wound healing. The consequence of the SASP is context dependent. Senescent cells secrete proinflammatory cytokines and growth factors known to enhance cell proliferation and transformation. Pro-angiogenic factors from senescent cells can promote tissue vascularization, while additional factors drive epithelial-to-mesenchymal transitions and increased invasiveness of premalignant cells.

development,[74] and the improved survival of animals following treatment with chemotherapy.[75] Of course, some senescent cells—for example, those occurring in benign melanocytic nevi—remain in tissues for reasons that are yet to be elucidated.

Perhaps counterintuitively, SASP factors previously appreciated for their protumorigenic activity contribute to tumor-suppressive aspects of the senescence response. For example, the proinflammatory cytokines interleukin-6 (IL6) and interleukin-8 (IL8) reinforce the senescence growth arrest, presumably by continuing to drive pro-proliferative and DDR signaling within the senescent cells.[76,77] The nuclear factor-κB (NFκB) family of transcription factors (master regulators of immune signaling traditionally regarded for their protumorigenic activity) is responsible for transcription of a substantial part of the SASP signature, including the expression of IL6 and IL8.[75,76,78,79]

Gene expression profiling indicates that NFκB controls the expression of more SASP genes during senescence than either p53 or RB, cementing its role as a key regulator of the senescence program.[75] During therapy-induced senescence, canonical NFκB activity was required both for the induction of senescence in tumor cells and for tumor regression following administration of chemotherapy, implying that the NFκB-driven SASP contributes to the senescent cell-intrinsic and -extrinsic aspects of the program and that NFκB can have remarkable antitumor activity.[75,78] These observations also suggest an important role for the SASP in facilitating chemotherapy responses, a possibility that is consistent with clinical data.[78]

Secreted factors are capable of influencing their microenvironment for as long as they persist. It is therefore possible that promitogenic SASP factors can promote the hyperproliferation and subsequent "bystander" or "secondary" senescence of normal and/or premalignant neighboring cells. In agreement, secondary senescence can be conferred on incubating normal growing cells in "conditioned" media from senescent cells.[80]

Alternatively, SASP factors might simultaneously drive the proliferation/invasiveness of neighboring malignant cells in which the senescence response is defective. For example, factors such as growth-related oncogene (GRO)-α and VEGF can facilitate the proliferation and transformation of premalignant cells[81] or promote tumor vascularization,[82] respectively. SASP factors can also promote invasiveness of premalignant cells.[79,83-85] Hence, in some circumstances, the continued presence of senescent cells (and thus production of SASP factors) in aged, damaged, and premalignant tissues may exacerbate the decline of tissue integrity and/or promote tumor development or relapse.[79,86,87] The precise contexts in which SASP factors are pro- or antitumorigenic remain to be determined.

Senescence and Noncancer Disease States

Because senescence limited the replicative capacity of cells in vitro, Hayflick and colleagues proposed that replicative senescence might generally reflect the process of aging at the cellular level.[2,3] Although there is perhaps little evidence to demonstrate that organismal aging occurs as a direct consequence of cellular aging, senescent cells accumulate in aging tissues at sites of age-related diseases, such as in atherosclerotic plaques, skin ulcers, and arthritic joints,[88,89] and expression of senescence markers such as $p16^{INK4A}$ increases in aging tissues.[90-92]

Consistent with an active contribution of senescent cells to aging, elimination of $p16^{INK4A}$-expressing cells from mouse tissues delays aging-related phenotypes such as sarcopenia, cataracts, and loss of adipose tissue.[93] Moreover, the late-life removal of $p16^{INK4A}$-expressing cells was sufficient to attenuate the progression of already established age-related disorders, suggesting that senescent cells contribute to the initiation and maintenance of the aging phenotype.[93] Although the etiology of the senescent cells in aging tissues is often unclear, at least some cases have been attributed to telomere dysfunction,[91,92] and experimental manipulations to produce telomere dysfunction can reproduce aging phenotypes.

Senescent cells may contribute to organismal aging in a number of ways. For example, because senescent cells are irreversibly arrested, they may be incapable of effectively repopulating aged/damaged tissue and thus promote tissue decline. At the same time, senescent cells remain metabolically active and have a SASP that may also influence the behavior of neighboring cells and promote tissue dysfunction.[94] They may also promote chronic inflammation, which is associated with diverse age-related diseases including cancer.[95] Conversely, senescence can positively facilitate wound-healing responses and promote the resolution of disease states, which may produce positive effects on tissue function. For example, in a murine model of chemical-induced liver fibrosis, the senescence program limited the extent of fibrosis by halting the proliferation of fibrogenic (activated stellate) cells, reducing extracellular matrix production, and promoting immune clearance of the senescent cells, suggesting a positive role in this wound-healing response. Other studies imply that senescence restricts fibrosis in cutaneous wound healing.[96] In both models, the SASP played a prominent role in the elimination of senescent cells and tissue restoration.

Emerging evidence thus suggests that the impact of senescence on tissue function may be influenced by acute versus chronic induction. Hence, senescence can facilitate tissue homeostasis following acute damage through a two-pronged mechanism, resulting in the proliferative arrest and changes to the microenvironment that lead to the elimination of damaged cells and the remodeling of the surrounding tissue to restore tissue structure and function. However, persistent cellular damage would continue to produce excessive presenescent or senescent cells that might contribute to tissue degeneration, the onset of age-related disease, or tumor formation.

Conclusions and Perspectives

The true physiological role for senescence appears to be the prevention of the propagation of damaged cells, where autonomous aspects control the proliferative arrest and the SASP reinforces arrest and further directs the immune system to promote their elimination. In a speculative model, the original evolutionary purpose of senescence might be to help coordinate wound-healing responses, and these actions may be co-opted, directly or indirectly, for tumor suppression. Ironically, when persistent, some of these activities may be protumorigenic and contribute to age-related pathologies.

Mechanistically, the program involves active interplay among at least three major transcription factors (Rb, p53, and NFκB) whose activities are known to play an important roles in cancer. Because senescence can be influenced by extracellular factors and molecules, for which drugs and inhibitors

are available, the possibilities for therapeutic modulation of the program in various settings exist. Such therapies could, in principle, harness the cell-autonomous aspects of the program to promote arrest or, alternatively, aid the immune recognition and clearance of senescent cells. Imperative for the success of such treatments is a thorough understanding of the molecules that govern senescence. Gaining new insight into senescence markers and biological aspects of the program in normal and pathological states is therefore of key importance for current and future biomedical research.

Key References

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. 1961;25:585-621.
7. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature*. 2003;426:194-198.
9. Shay JW, Wright WE. Telomeres and telomerase in normal and cancer stem cells. *FEBS Lett*. 2010;584:3819-3825.
11. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science*. 1998;279:349-352.
19. Shay JW, Pereira-Smith OM, Wright WE. A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res*. 1991;196:33-39.
20. Gu W, Putral L, McMillan N. siRNA and shRNA as anticancer agents in a cervical cancer model. *Methods Mol Biol*. 2008;442:159-172.
21. Hall AH, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. *J Virol*. 2003;77:6066-6069.
22. Horner SM, DeFilippis RA, Manuelidis L, et al. Repression of the human papillomavirus E6 gene initiates p53-dependent, telomerase-independent senescence and apoptosis in HeLa cervical carcinoma cells. *J Virol*. 2004;78:4063-4073.
23. Johung K, Goodwin EC, DiMaio D. Human papillomavirus E7 repression in cervical carcinoma cells initiates a transcriptional cascade driven by the retinoblastoma family, resulting in senescence. *J Virol*. 2007;81:2102-2116.
25. Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997;88:593-602.
28. Mallette FA, Gaumont-Leclerc MF, Ferbeyre G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev*. 2007;21:43-48.
29. Rodier F, Coppe JP, Patil CK, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol*. 2009;11:973-979.
30. Di Micco R, Sulli G, Dobreva M, et al. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat Cell Biol*. 2011;13:292-302.
31. Courtois-Cox S, Genther Williams SM, Reczek EE, et al. A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell*. 2006;10:459-472.
32. Sarkisian CJ, Keister BA, Stairs DB, et al. Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol*. 2007;9:493-505.
33. Tuveson DA, Shaw AT, Willis NA, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*. 2004;5:375-387.
34. Schmitt CA, Fridman JS, Yang M, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell*. 2002;109:335-346.
36. Roberson RS, Kussick SJ, Vallieres E, et al. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res*. 2005;65:2795-2803.
37. Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. *Nature*. 2005;436:642.
38. Braig M, Lee S, Loddenkemper C, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*. 2005;436:660-665.
39. Lazzerini Denchi E, Attwooll C, Pasini D, et al. Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol*. 2005;25:2660-2672.
40. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005;436:725-730.
41. Majumder PK, Grisanzio C, O'Connell F, et al. A prostatic intraepithelial neoplasia-dependent p27 Kip1 checkpoint induces senescence and inhibits cell proliferation and cancer progression. *Cancer Cell*. 2008;14:146-155.
42. Bennecke M, Kriegl L, Bajbouj M, et al. Ink4a/Arf and oncogene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis. *Cancer Cell*. 2010;18:135-146.
43. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436:720-724.
44. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19-20.
45. Poynter JN, Elder JT, Fullen DR, et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res*. 2006;16:267-273.
46. Rother J, Jones D. Molecular markers of tumor progression in melanoma. *Curr Genomics*. 2009;10:231-239.
47. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer*. 2008;8:671-682.
56. Sage J, Miller AL, Perez-Mancera PA, et al. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature*. 2003;424:223-228.
57. Narita M, Nunez S, Heard E, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell*. 2003;113:703-716.
58. Chicas A, Wang X, Zhang C, et al. Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell*. 2010;17:376-387.
59. Larsson LG. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. *Semin Cancer Biol*. 2011;21:367-376.
60. Zhang R, Chen W, Adams PD. Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol*. 2007;27:2343-2358.
61. Chicas A, Kapoor A, Wang X, et al. H3K4 demethylation by Jarid1a and Jarid1b contributes to retinoblastoma-mediated gene silencing during cellular senescence. *Proc Natl Acad Sci U S A*. 2012;109:8971-8976.
63. Xue W, Zender L, Miething C, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*. 2007;445:656-660.
64. Ventura A, Kirsch DG, McLaughlin ME, et al. Restoration of p53 function leads to tumour regression in vivo. *Nature*. 2007;445:661-665.
65. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell*. 2006;127:1323-1334.
68. de Stanchina E, Querido E, Narita M, et al. PML is a direct p53 target that modulates p53 effector functions. *Mol Cell*. 2004;13:523-535.
69. Vernier M, Bourdeau V, Gaumont-Leclerc MF, et al. Regulation of E2Fs and senescence by PML nuclear bodies. *Genes Dev*. 2011;25:41-50.
70. Aksoy O, Chicas A, Zeng T, et al. The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. *Genes Dev*. 2012;26:1546-1557.
71. Carvajal LA, Hamard PJ, Tonnessen C, et al. E2F7, a novel target, is upregulated by p53 and mediates DNA damage-dependent transcriptional repression. *Genes Dev*. 2012;26:1533-1545.
72. Krizhanovsky V, Yon M, Dickins RA, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell*. 2008;134:657-667.
73. Sagiv A, Biran A, Yon M, et al. Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene*. 2013 Apr 11;32(15):1971-1977. doi: 10.1038/onc.2012.206. Epub 2012 Jul 2.
74. Kang TW, Yevsa T, Woller N, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature*. 2011;479:547-551.

75. Chien Y, Scuoppo C, Wang X, et al. Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes Dev*. 2011;25:2125-2136.
76. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133:1006-1018.
77. Kuilman T, Michaloglou C, Vredeveld LC, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*. 2008;133:1019-1031.
78. Jing H, Kase J, Dorr JR, et al. Opposing roles of NF-kappaB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev*. 2011;25:2137-2146.
79. Ohanna M, Giuliano S, Bonet C, et al. Senescent cells develop a PARP-1 and nuclear factor-κB-associated secretome (PNAS). *Genes Dev*. 2011;25:1245-1261.
80. Wajapeyee N, Serra RW, Zhu X, et al. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*. 2008;132:363-374.
81. Coppe JP, Patil CK, Rodier F, et al. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One*. 2010;5:e9188.
82. Coppe JP, Kauser K, Campisi J, et al. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem*. 2006;281:29568-29574.
83. Liu D, Hornsby PJ. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res*. 2007;67:3117-3126.
85. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. 2008;6:2853-2868.
86. Jackson JG, Pant V, Li Q, et al. p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. *Cancer Cell*. 2012;21:793-806.
87. Canino C, Mori F, Cambria A, et al. SASP mediates chemoresistance and tumor-initiating-activity of mesothelioma cells. *Oncogene*. 2012;31:3148-3163.
91. Herbig U, Ferreira M, Condel L, et al. Cellular senescence in aging primates. *Science*. 2006;311:1257.
92. Jeyapalan JC, Ferreira M, Sedivy JM, et al. Accumulation of senescent cells in mitotic tissue of aging primates. *Mech Ageing Dev*. 2007;128:36-44.
93. Baker DJ, Wijshake T, Tchkonia T, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature*. 2011;479:232-236.
94. Adams PD. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Mol Cell*. 2009;36:2-14.
95. Freund A, Orjalo AV, Desprez PY, et al. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med*. 2010;16:238-246.
96. Jun JI, Lau LF. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat Cell Biol*. 2010;12:676-685.

References

1. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. 1961;25:585-621.
2. Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res*. 1965;37:614-636.
3. Smith JR, Hayflick L. Variation in the life-span of clones derived from human diploid cell strains. *J Cell Biol*. 1974;62:48-53.
4. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature*. 1990;345:458-460.
5. McEachern MJ, Krauskopf A, Blackburn EH. Telomeres and their control. *Annu Rev Genet*. 2000;34:331-358.
6. Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science*. 2002;295:2446-2449.
7. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature*. 2003;426:194-198.
8. Shay JW, Wright WE, Werbin H. Defining the molecular mechanisms of human cell immortalization. *Biochim Biophys Acta*. 1991;1072:1-7.
9. Shay JW, Wright WE. Telomeres and telomerase in normal and cancer stem cells. *FEBS Lett*. 2010;584:3819-3825.
10. Blackburn EH. Telomerases. *Annu Rev Biochem*. 1992;61:113-129.
11. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. *Science*. 1998;279:349-352.
12. Harley CB. Telomerase is not an oncogene. *Oncogene*. 2002;21:494-502.
13. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science*. 1994;266:2011-2015.
14. Hahn WC, Counter CM, Lundberg AS, et al. Creation of human tumour cells with defined genetic elements. *Nature*. 1999;400:464-468.
15. Todaro GJ, Green H. High frequency of SV40 transformation of mouse cell line 3T3. *Virology*. 1966;28:756-759.
16. Ruley HE. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature*. 1983;304:602-606.
17. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*. 1983;304:596-602.
18. Shay JW, Wright WE. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 large T-antigen. *Exp Cell Res*. 1989;184:109-118.
19. Shay JW, Pereira-Smith OM, Wright WE. A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res*. 1991;196:33-39.
20. Gu W, Putral L, McMillan N. siRNA and shRNA as anticancer agents in a cervical cancer model. *Methods Mol Biol*. 2008;442:159-172.
21. Hall AH, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. *J Virol*. 2003;77:6066-6069.
22. Horner SM, DeFilippis RA, Manuelidis L, et al. Repression of the human papillomavirus E6 gene initiates p53-dependent, telomerase-independent senescence and apoptosis in HeLa cervical carcinoma cells. *J Virol*. 2004;78:4063-4073.
23. Johung K, Goodwin EC, DiMaio D. Human papillomavirus E7 repression in cervical carcinoma cells initiates a transcriptional cascade driven by the retinoblastoma family, resulting in senescence. *J Virol*. 2007;81:2102-2116.
24. Wei S, Sedivy JM. Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts. *Cancer Res*. 1999;59:1539-1543.
25. Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997;88:593-602.
26. Lin AW, Barradas M, Stone JC, et al. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev*. 1998;12:3008-3019.
27. Zhu J, Woods D, McMahon M, et al. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev*. 1998;12:2997-3007.
28. Mallette FA, Gaumont-Leclerc MF, Ferbeyre G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev*. 2007;21:43-48.
29. Rodier F, Coppe JP, Patil CK, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol*. 2009;11:973-979.
30. Di Micco R, Sulli G, Dobreva M, et al. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat Cell Biol*. 2011;13:292-302.
31. Courtois-Cox S, Genther Williams SM, Reczek EE, et al. A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell*. 2006;10:459-472.
32. Sarkisian CJ, Keister BA, Stairs DB, et al. Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol*. 2007;9:493-505.
33. Tuveson DA, Shaw AT, Willis NA, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*. 2004;5:375-387.
34. Schmitt CA, Fridman JS, Yang M, et al. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell*. 2002;109:335-346.
35. te Poele RH, Okorokov AL, Jardine L, et al. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. *Cancer Res*. 2002;62:1876-1883.
36. Roberson RS, Kussick SJ, Vallieres E, et al. Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res*. 2005;65:2795-2803.
37. Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. *Nature*. 2005;436:642.
38. Braig M, Lee S, Loddenkemper C, et al. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*. 2005;436:660-665.
39. Lazzerini Denchi E, Attwooll C, Pasini D, et al. Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol*. 2005;25:2660-2672.
40. Chen Z, Trotman LC, Shaffer D, et al. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*. 2005;436:725-730.
41. Majumder PK, Grisanzio C, O'Connell F, et al. A prostatic intraepithelial neoplasia-dependent p27 Kip1 checkpoint induces senescence and inhibits cell proliferation and cancer progression. *Cancer Cell*. 2008;14:146-155.
42. Bennecke M, Kriegl L, Bajbouj M, et al. Ink4a/Arf and oncogene-induced senescence prevent tumor progression during alternative colorectal tumorigenesis. *Cancer Cell*. 2010;18:135-146.
43. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436:720-724.
44. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19-20.
45. Poynter JN, Elder JT, Fullen DR, et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. *Melanoma Res*. 2006;16:267-273.
46. Rother J, Jones D. Molecular markers of tumor progression in melanoma. *Curr Genomics*. 2009;10:231-239.
47. Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer*. 2008;8:671-682.
48. Bates S, Phillips AC, Clark PA, et al. p14ARF links the tumour suppressors RB and p53. *Nature*. 1998;395:124-125.
49. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta*. 2002;1602:73-87.
50. Sharpless NE, Bardeesy N, Lee KH, et al. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature*. 2001;413:86-91.
51. Kamijo T, Zindy F, Roussel MF, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell*. 1997;91:649-659.
52. Mulligan G, Jacks T. The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet*. 1998;14:223-229.
53. Lee MH, Williams BO, Mulligan G, et al. Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev*. 1996;10:1621-1632.
54. Cobrinik D, Lee MH, Hannon G, et al. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev*. 1996;10:1633-1644.
55. Sage J, Mulligan GJ, Attardi LD, et al. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev*. 2000;14:3037-3050.

56. Sage J, Miller AL, Perez-Mancera PA, et al. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature*. 2003;424:223-228.
57. Narita M, Nunez S, Heard E, et al. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell*. 2003;113:703-716.
58. Chicas A, Wang X, Zhang C, et al. Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell*. 2010;17:376-387.
59. Larsson LG. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. *Semin Cancer Biol*. 2011;21:367-376.
60. Zhang R, Chen W, Adams PD. Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol*. 2007;27:2343-2358.
61. Chicas A, Kapoor A, Wang X, et al. H3K4 demethylation by Jarid1a and Jarid1b contributes to retinoblastoma-mediated gene silencing during cellular senescence. *Proc Natl Acad Sci U S A*. 2012;109:8971-8976.
62. Harvey M, Sands AT, Weiss RS, et al. In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*. 1993;8:2457-2467.
63. Xue W, Zender L, Miething C, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*. 2007;445:656-660.
64. Ventura A, Kirsch DG, McLaughlin ME, et al. Restoration of p53 function leads to tumour regression in vivo. *Nature*. 2007;445:661-665.
65. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell*. 2006;127:1323-1334.
66. Pantoja C, Serrano M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene*. 1999;18:4974-4982.
67. Ferbeyre G, de Stanchina E, Querido E, et al. PML is induced by oncogenic ras and promotes premature senescence. *Genes Dev*. 2000;14:2015-2027.
68. de Stanchina E, Querido E, Narita M, et al. PML is a direct p53 target that modulates p53 effector functions. *Mol Cell*. 2004;13:523-535.
69. Vernier M, Bourdeau V, Gaumont-Leclerc MF, et al. Regulation of E2Fs and senescence by PML nuclear bodies. *Genes Dev*. 2011;25:41-50.
70. Aksoy O, Chicas A, Zeng T, et al. The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. *Genes Dev*. 2012;26:1546-1557.
71. Carvajal LA, Hamard PJ, Tonnessen C, et al. E2F7, a novel target, is upregulated by p53 and mediates DNA damage-dependent transcriptional repression. *Genes Dev*. 2012;26:1533-1545.
72. Krizhanovsky V, Yon M, Dickins RA, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell*. 2008;134:657-667.
73. Sagiv A, Biran A, Yon M, et al. Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene*. 2013 Apr 11;32(15):1971-1977. doi: 10.1038/onc.2012.206. Epub 2012 Jul 2.
74. Kang TW, Yevsa T, Woller N, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature*. 2011;479:547-551.
75. Chien Y, Scuoppo C, Wang X, et al. Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes Dev*. 2011;25:2125-2136.
76. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133:1006-1018.
77. Kuilman T, Michaloglou C, Vredeveld LC, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*. 2008;133:1019-1031.
78. Jing H, Kase J, Dorr JR, et al. Opposing roles of NF-kappaB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev*. 2011;25:2137-2146.
79. Ohanna M, Giuliano S, Bonet C, et al. Senescent cells develop a PARP-1 and nuclear factor-κB-associated secretome (PNAS). *Genes Dev*. 2011;25:1245-1261.
80. Wajapeyee N, Serra RW, Zhu X, et al. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*. 2008;132:363-374.
81. Coppe JP, Patil CK, Rodier F, et al. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One*. 2010;5:e9188.
82. Coppe JP, Kauser K, Campisi J, et al. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem*. 2006;281:29568-29574.
83. Liu D, Hornsby PJ. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res*. 2007;67:3117-3126.
84. Krtolica A, Parrinello S, Lockett S, et al. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A*. 2001;98:12072-12077.
85. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. 2008;6:2853-2868.
86. Jackson JG, Pant V, Li Q, et al. p53-mediated senescence impairs the apoptotic response to chemotherapy and clinical outcome in breast cancer. *Cancer Cell*. 2012;21:793-806.
87. Canino C, Mori F, Cambria A, et al. SASP mediates chemoresistance and tumor-initiating-activity of mesothelioma cells. *Oncogene*. 2012;31:3148-3163.
88. Krtolica A, Campisi J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. *Int J Biochem Cell Biol*. 2002;34:1401-1414.
89. Minamino T, Komuro I. Role of telomere in endothelial dysfunction in atherosclerosis. *Curr Opin Lipidol*. 2002;13:537-543.
90. Zindy F, Quelle DE, Roussel MF, et al. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene*. 1997;15:203-211.
91. Herbig U, Ferreira M, Condel L, et al. Cellular senescence in aging primates. *Science*. 2006;311:1257.
92. Jeyapalan JC, Ferreira M, Sedivy JM, et al. Accumulation of senescent cells in mitotic tissue of aging primates. *Mech Ageing Dev*. 2007;128:36-44.
93. Baker DJ, Wijshake T, Tchkonia T, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature*. 2011;479:232-236.
94. Adams PD. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Mol Cell*. 2009;36:2-14.
95. Freund A, Orjalo AV, Desprez PY, et al. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med*. 2010;16:238-246.
96. Jun JI, Lau LF. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat Cell Biol*. 2010;12:676-685.

Cyrus M. Ghajar, Ana Luísa Correia, and Mina J. Bissell

16

The Role of the Microenvironment in Tumor Initiation, Progression, and Metastasis

The discovery in the 1970s of proto-oncogenes, genes that become oncogenic ("cancer causing") either through genetic modifications or increased expression, and tumor suppressor genes, those that if expressed at the right levels would suppress progression to malignancy, spurred a revolution. Given the excitement and the implication of these discoveries, it may not be surprising that most cancer researchers never looked back. Thus much of the work of the early cancer research pioneers such as Paget, Rous, Warburg, and Berenblum (see later discussion) that drew attention to other aspects of cancer became unpopular and were considered beside the point.

The subsequent decades brought technologies that enabled automated sequencing of DNA, which eventually made the dream of sequencing whole organismal and tumor genomes a reality. The hope was that pinpointing aberrations in genetic sequence would allow one to understand the origins of cancer.[1] Dealing with the mutations by fixing the genes through gene therapy or neutralizing the gene products would thus provide a viable cure. However, the picture that emerges 40 years later is much more complex.[2] For breast cancer, we know now that the frequency of somatic mutations exceeds 1 per 1 million DNA base pairs.[3] We also know that a single tumor may have hundreds of mutations,[3] that some mutations *(TP53, PIK3CA, GATA3)* are more prevalent than others,[4] but that even these are not present in the majority of patients.[4] Given this enormous complexity, where would we start?

This approach has also taught us a lot about what the genome may not be able to tell us. For instance, autopsy studies have revealed that the fraction of individuals harboring neoplastic lesions within their breast or prostate is 27- to 142-fold higher than the actual incidence rates of breast and prostate cancer.[7,8] If the initial mutation was sufficient to cause cancer, why do the great majority of these neoplasms fail to progress to frank carcinomas? Another aspect of tumor progression that cannot be explained solely by genomic aberrations is why tumors metastasize when they do. The prevailing hypothesis had been that metastases reflect the pinnacle of tumor evolution: tumor cells would have to acquire a set of mutations in order to disseminate from the primary tumor to another tissue.[9] Now, it is clear that tumor cells disseminate very early during tumor progression despite few genetic abnormalities[10-12] and that these tumors may emerge even faster than the primary tumor itself (this is referred to as *cancer of unknown primary*). So, if metastatic outgrowth does not require additional mutations from the primary tumor, what drives the metastatic program? Other questions along these lines are outlined elsewhere.[13]

The need to answer such questions has spawned a newfound appreciation that the complexity that governs tumor phenotype cannot be explained only at the genetic level. As a result, the focus has slowly begun to shift to the study of the tumor's microenvironment. Whereas this appreciation may be newfound, the concept of the microenvironment's importance is not (see Figure 16-1 for a timeline).[14-17]

Half of the secret of the cell is outside the cell: a historical perspective on the role of the microenvironment

In 1889, Stephen Paget published results of an autopsy study he conducted on 735 breast cancer patients. His study revealed that these patients tended to have metastases within four tissues: lung, liver, uterus, and bone. Empowered by these observations, as well as those of peers such as Langenbeck and Fuchs, Paget formulated his now-famous "seed and soil" hypothesis: "Every single cancer cell must be regarded as an organism, alive and capable of development. When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil."[18] Perhaps this is where an appreciation for

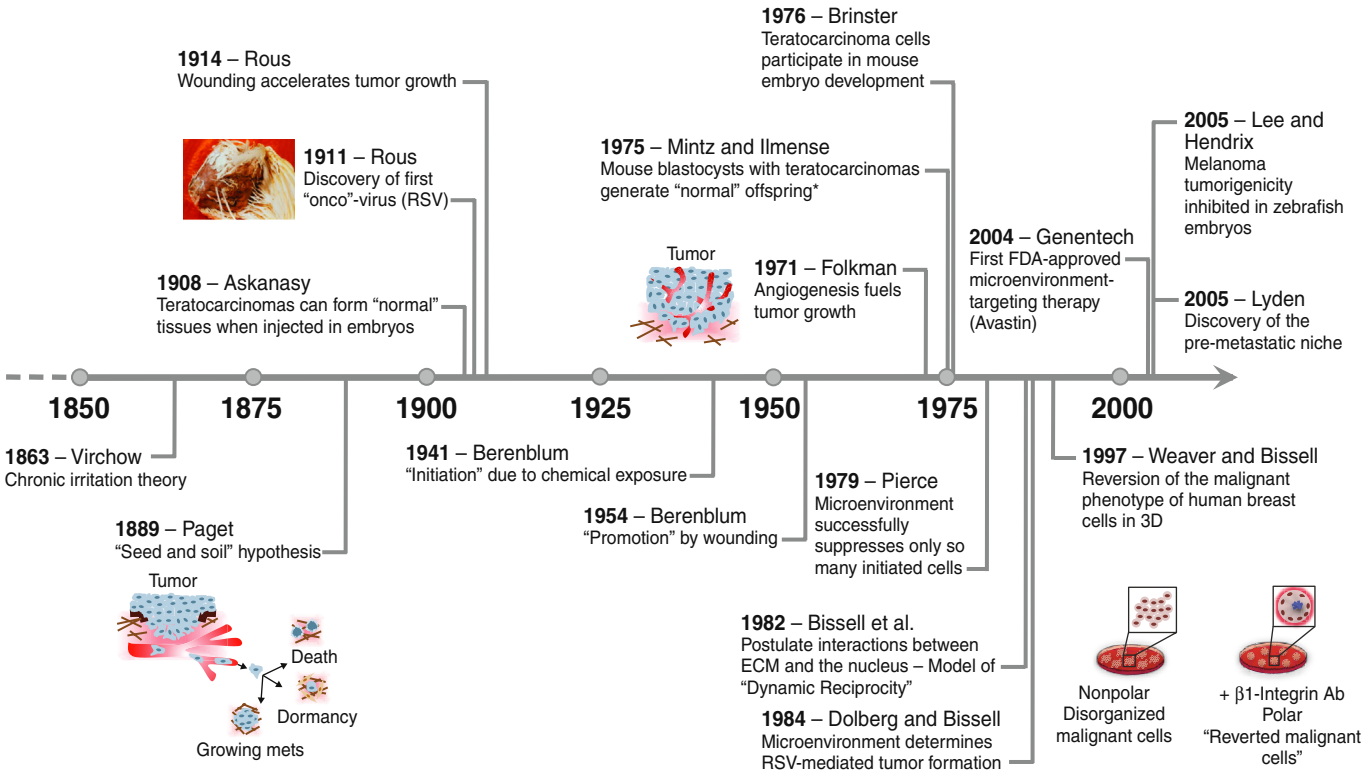


Figure 16-1 A historical perspective on the role of the microenvironment in cancer A timeline detailing landmark discoveries that showed the dominance of microenvironment over genotype. If space had permitted, a number of other important experiments could have been included, such as Emerman and Pitelka's demonstration that normal mammary epithelial cells on floating collagen gels recapitulate their in vivo phenotype,[14] Folkman's demonstration that cell shape regulates DNA synthesis,[15] and experiments demonstrating that reconstituted 3D BM gels act as a "blotter" to distinguish the normal from the malignant phenotype.[16,17]

the microenvironment began. Remarkably, the enduring interpretation of Paget's hypothesis is that certain soils are favorable for tumor growth. This has indeed dominated the landscape of metastasis research and is the subject of the following subsection (Promoting Microenvironments). Inherent in Paget's message, however, was that certain soils are inhospitable to a tumor's seed. In the same article, Paget remarks on a colleague's interpretation that instead of a predisposition to receive a seed, certain organs exhibit "diminished resistance."[18] Thus the second subsection (Suppressive Microenvironments) deals with this idea.

Promoting Microenvironments

What makes a given microenvironment favorable for the growth of a tumor cell is a topic that is germane to all tumors, disseminated or not. For instance, the probability of harboring an occult (i.e., hidden) neoplasm increases with age to nearly 100% in an organ like the thyroid gland, yet only 0.1% of the population is ever diagnosed with thyroid cancer.[19] What allows the emergence of tumors in some, but not others, is a question that has been pursued since the turn of the 20th century. Peyton Rous and others concluded that a transplanted tumor would not take unless there was a stromal reaction and immediate vascularization of the implant.[20] These properties are common to both inflammation and wounding, and each has long been suspected of creating a tumor-promoting microenvironment. As early as 1863, Virchow noted that chronic irritation and prior injury could precondition tissue for tumor formation.[21] Rous was among the first to show this conclusively by demonstrating that wounding the peritoneal cavities of mice inoculated with tumor cells accelerated the growth of tumors within their visceral organs.[22]

Further evidence for the tumor-promoting power of the wounding microenvironment came from the vast literature on chemical carcinogens. An extensive body of work established that chemicals within coal tar such as benzo[*a*]pyrene derivatives, despite being known mutagens, were not sufficient to cause skin cancer on their own. Despite "initiation" due to chemical exposure, normal skin guards against progression unless the carcinogen dose is so excessive that it damages the tissue in addition to causing mutagenesis.[23,24] This second step, called *promotion*, is required and is generally caused by wounding or by other toxic agents, many of which are associated with aberrant tissue repair and fibrosis.[25-27] The discovery of the first "onco"-virus, referred to as *Rous sarcoma virus* (RSV),[28] later provided some of the most conclusive evidence that wounding promotes tumor

formation. In discovering RSV, Rous took the filtrate of a chicken tumor and noted that this cell-free filtrate would induce sarcomas in recipient chickens,[28] thus proving Koch's postulate. Decades later, when experimenting with RSV, Bissell and colleagues noted that the injected virus circulated throughout the bird, but tumors tended to arise only at the injection site.[29] Was the wound created by the injection needle the key factor? Nicking the contralateral wing of infected chickens caused tumors to arise also at the sites of abrasions.[29] This phenomenon was mediated by transforming growth factor (TGF)-β1, which was expressed at the site of injection shortly after wounding and shown to induce tumors on its own even in the absence of wounding.[30]

Of course, processes such as wounding and fibrosis are inextricably linked with the formation of new vasculature (e.g., through angiogenesis), but it was not until Judah Folkman's work in the early 1970s that a causal relationship between tumor growth and angiogenesis was established. Tumor fragments or tumor cells grafted onto the rabbit cornea were observed to induce sprouting from existing vasculature as they grew.[31] Physically preventing microvasculature from reaching the implant resulted in a latent mass where tumor cell proliferation was countered by apoptosis. Folkman's work demonstrated, for the first time, a nontumor cell—the endothelial cell—that was critical to the growth of a tumor. His work also started a new field focused on "anti-angiogenesis" based on Folkman's hypothesis: "Solid tumors can grow to visibility only if they can vascularize themselves. Therefore, the mechanism by which tumor implants stimulate neovascularization must be well understood before therapy based upon interference with angiogenesis can be devised."[32] The angiogenesis inhibitor bevacizumab (Avastin) would become the first therapy explicitly targeting the microenvironment approved by the United States FDA (2004) (see Chapter 17 for more information).

Suppressive Microenvironments

The studies just described established key roles for the microenvironment in promoting tumor growth, which is the primary focus of this chapter. However, it is worth mentioning that much of the milestone research in demonstrating the importance of the microenvironment did so by showing that context could override tumorigenicity—that is, tumor cells could be tricked into thinking they are normal if provided the right cues. The observation that the embryo comprises such a suppressive microenvironment is one that was first made more than 100 years ago, when Askanazy showed that ovarian teratomas could form "normal" tissues composed of the correct embryonic germinal layers when injected into embryos.[33] Decades later, a series of studies from different laboratories provided further evidence that the embryonic microenvironment could induce tumors to function normally in development. Mintz and Illmensee reported to have injected embryonic teratoma core cells from mice with a steel coat genotype into blastocysts from C57-b/b mice (which have black coats). The blastocysts gave rise to functionally "normal" offspring.[34] Their next paper[35] reported that the mice born from the initial experiments produced an offspring that was completely normal and had a mosaic (i.e., striped) coat, implying that the teratocarcinoma could pass through the germline. Although it is true that this work has not been confirmed in other laboratories, there are some dramatic variations on the theme. For instance, Brinster injected two to four teratocarcinoma cells from agouti brown mice into 4-day-old blastocysts of Swiss albino mice. One of the 60 injected mice retained the teratocarcinoma cells (based on the presence of brown hair patches on the white mouse) and proceeded to develop normally as well.[36] Pierce later essentially quantified the balance of power between the embryonic microenvironment and the malignant cell by demonstrating that the embryonic microenvironment could suppress the malignant phenotype of one to a few implanted tumor cells, but that this ability diminished as the number of injected tumor cells increased.[37] Perhaps this offers a hint that our bodies are able to successfully suppress only so many initiated cells, and that this power diminishes with age.

The suppressive effect of the embryonic microenvironment was demonstrated in species other than mice as well. Using RSV, Dolberg and Bissell showed conclusively that cells within injected chick embryos expressed the virus, but early embryos failed to form tumors.[38] Maintaining embryonic architecture was key, however, as dissociating the embryos and placing the $PP60^{src}$-marked cells (using *LacZ*) in culture resulted in rapid transformation of the blue-labeled cells.[39] Hendrix and colleagues more recently reported similar findings for aggressive melanoma cells injected into zebrafish embryos.[40] The lasting impact of these studies is that tissue architecture is dominant even to a powerful oncogene in embryos. These studies also offered the clue that the malignant genotype could be overridden if somehow provided with suppressive cues from the microenvironment.

Taking advantage of this insight required an assay that would allow normal and malignant cells to recapitulate their in vivo phenotypes in culture. This was achieved by Petersen, Bissell, and colleagues culturing cells in a three-dimensional (3D) reconstituted basement membrane (BM) gel. In 3D but not 2D conditions, primary mammary epithelial cells or nonmalignant cell lines formed growth-arrested, polarized acini resembling terminal ductal lobular units of the breast, whereas malignant cells formed disorganized masses that continued to grow.[16,17] By examining the expression profiles of integrins—heterodimeric receptors on the cellular surface that transduce signals from the extracellular matrix (ECM) through traditional and nontraditional pathways

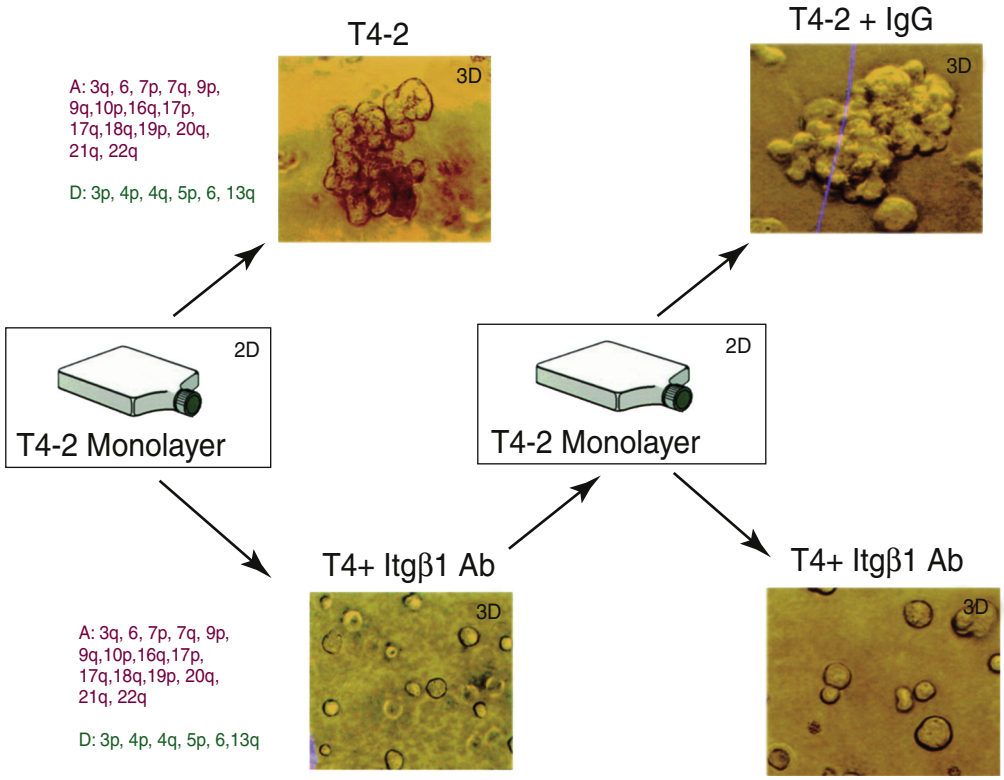


Figure 16-2 Serial passaging from 3D to 2D cultures (and back) demonstrates phenotypic reversion as opposed to selection Phase contrast micrographs of T4-2 cells grown in the presence of mock antibody (IgG) or anti–β1-integrin function blocking antibody (Itgb1 Ab) within 3D reconstituted basement membrane gels. Despite two rounds of treatment, reverted cells were able to resume their original tumorigenic phenotypes after being passaged and recultured in the absence of antibody. Note that despite displaying a nonmalignant phenotype, reverted tumor cells retain an aberrant genome (genomic amplifications [*A*] shown in *red*, deletions [*D*] shown in *green*). *(Figure adapted from Weaver VM, Petersen OW, Wang F, et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies.* J Cell Biol. *1997;137:231-245.)*

to alter gene expression—Weaver, Bissell, and colleagues noted aberrant overexpression of integrins and a number of other receptors such as EGFR on malignant cells.[41] Suspecting that these receptors were key nodes that integrated signals from the microenvironment to direct cell behavior, the authors began to restore levels of aberrant receptors to normal levels, starting by applying inhibitory antibodies targeting integrin β1 in malignant cells cultured in 3D gels. The result was a dramatic "phenotypic reversion" of malignant breast epithelial cells to structures that looked and behaved like their normal counterparts.[41] To show that this treatment was not somehow selecting for a nonmalignant subpopulation of cells, Weaver and colleagues dissociated tumor cell clusters from 3D gels, replated them onto plastic, and then passaged them back into 3D gels in the absence of blocking antibody. Tumor cells once again formed malignant clusters (Figure 16-2).[41] This strategy led to the discovery of a host of signaling molecules that act in concert to regulate/integrate epithelial phenotype. Many of these molecules effect interactions between cells and their microenvironments, including ECM molecules,[42] growth/ECM receptors,[43,44] and matrix metalloproteinases (MMPs) that digest ECM components.[45] Remarkably, targeting only one of these aberrantly expressed molecules restores the levels of all the others back to normal, demonstrating the potential of normalizing aberrant microenvironmental signaling to redirect entire signaling webs and impair manifestation of the malignant genotype.[13,46] It is critical to re-emphasize that these pathways integrate only in 3D; even something as seemingly well-understood as glucose metabolism ties into these noncanonical pathways and regulates malignancy only when interrogated in context.[47]

The Tumor Organ

The historical studies detailed above demonstrate that a tumor is a product of aberrant genomes interacting with an enabling microenvironment. This concept is easier to appreciate if one considers the tumor as a dysfunctional organ.[48] On a basic level, an organ has the following properties:

1. Organs are multicellular and are composed of multiple tissue layers. Functional tissue layers consist of epithelia, which are tubelike structures that carry fluid, and epithelia are separated from surrounding stroma by a specialized ECM called the basement membrane (BM).

2. Organs are governed by properties that emerge as a result of the interactions between the cells, ECM molecules, and soluble factors composing the organ, and the sum of these interactions is greater than any one of the individual parts.
3. Organs have unique signatures of functional differentiation; for instance, the mammary gland produces milk, the pancreas produces insulin, and bone marrow is responsible for maintaining homeostasis of the hematopoietic and lymphatic systems.

Although tumors lack proper function, they do have the first two of these traits in common with organs. The focus of this section is on the second of these traits: the properties that emerge as a result of a tumor's interactions with its microenvironment. There are two ways to illustrate this concept. The first would be to consider the parallels between a developing organ and a developing tumor. The scope of this discussion would extend well beyond this chapter, however, because just as each organ develops differently, tumors of these organs develop distinctly as well. Instead, a more general way to illustrate the concept of the tumor organ is to consider what happens when an organ is injured—it attempts to heal. The wounding microenvironment shares a great deal in common with the tumor microenvironment (illustrated in Figure 16-3), the difference being that wounds eventually stop healing, whereas a tumor's microenvironment persists. This is why tumors have been called "wounds that do not heal."[49]

The Wounding Microenvironment

With a wound, the goal is to restore function of injured tissue, which means that damaged epithelia must be resealed. Compromised blood vessels leak plasma and platelets into the wound site (or simply hemorrhage blood because of more serious injury).[48,50] Tissue procoagulants such as tissue factor initiate a cascade resulting in a clot, rich in the ECM molecules fibrin and fibronectin, which entraps platelets and blood cells. Platelets are a rich initial source of clotting factors, mitogens, and chemoattracting cues that lure cells into the wound site. Inflammatory cells are among these cells, releasing extracellular proteinases such as MMPs and cysteine cathepsins that cleave the provisional ECM to facilitate migration. Fibroblasts follow and become activated by TGF-β and fibronectin splice variants within the clot to become myofibroblasts—muscle-like cells with an enhanced ability to exert contractile force.[51,52] These cells are charged with cinching the wound, and in doing so deposit copious amounts of ECM, consisting primarily of type I collagen (Col-1), to scaffold the tissue in its contracted state.[50] Endothelial cells are stimulated to invade from nearby microvasculature by angiogenic factors (e.g., VEGF, FGF-2) secreted by platelets, immune cells, and fibroblasts, and also released from the provisional ECM by MMPs (e.g., MMP-9), to rapidly vascularize and feed the new tissue.[53] Meanwhile, this complex cascade of events reduces adhesiveness of adjacent epithelial cells, which undergo an epithelial-mesenchymal transition (EMT) in order to migrate to reseal the epithelium. The cells will later revert back to their normal state by redepositing BM, reengaging each other through cell-cell junctions, growth arresting, and functionally differentiating.[48]

Thus, the wounding microenvironment is populated by circulating cell types such as immune cells and platelets, and resident tissue cells such as fibroblasts and endothelial cells. These cellular constituents engage in a dynamic and reciprocal chorus via secreted factors and ECM molecules to activate nearby epithelial cells to close the wound via proliferation and migration. The biggest difference between a tumor and the healing wound is what happens next. In the proper wounding context, not only does the epithelium differentiate, but activated cell types do not persist, the provisional ECM is remodeled, and secretion of stimulatory growth factors and cytokines is dampened. There is still much to be learned about what comprises this "homeostatic switch"—that is, the cues that tell a tissue to stop remodeling, presumably because it is now fully functional. For instance, what exactly happens to all of the activated myofibroblasts is not known. They may deactivate, transdifferentiate, or undergo apoptosis (this fails to occur in individuals with chronic wounding disorders, and it should not surprise the reader that these individuals are also more susceptible to cancer[54]). The stimuli that cause myofibroblasts to undergo any of these programs are also unknown. Perhaps uncovering these cues will offer insight as to how the tumor microenvironment itself can be phenotypically reverted so that it does not persist.

The Tumor Microenvironment

Whether a tumor creates its own microenvironment or the aberrant microenvironment causes a tumor is a conundrum of tumor biology. There is evidence for both. Regardless, what manifests is a dynamic interplay between a tumor and its surroundings that ultimately results in loss of organ structure and architecture. Loss of architecture is a hallmark of cancer that reflects behaviors such as deregulated growth, enhanced survival, new blood vessel formation, stromal activation, and inappropriate migratory and invasive behavior of cells.[55] The microenvironment becomes a runaway train, and activation is heightened as entropy (loss of cell and tissue architecture) increases. As the reader goes through the remainder of this chapter, keep the following questions in mind: at what stage of progression do tumor cells or cells in

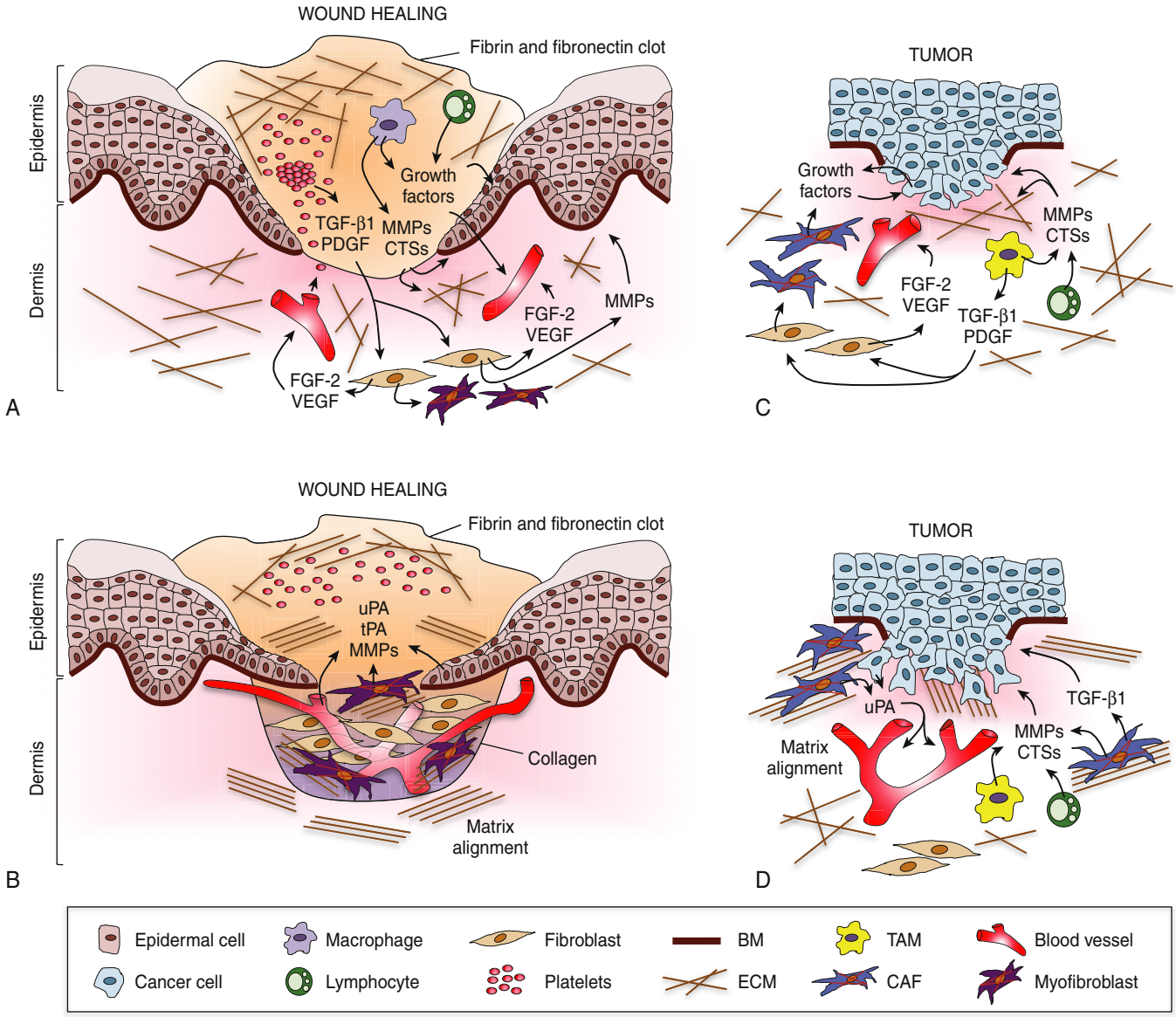


Figure 16-3 Tumors: "Wounds that do not heal" **(A)** Immediately after injury, damaged blood vessels leak plasma and platelets, which form a hemostatic plug and release vasoactive mediators to increase vascular permeability and enable the influx of serum fibrinogen to generate a fibrin and fibronectin-rich clot. Platelets produce chemotactic factors such as TGF-β and PDGF, which lure inflammatory cells and fibroblasts into the wound site. These cells produce extracellular proteases, including matrix metalloproteinases (MMPs) and cathepsins (CTSs), which remodel extracellular matrix (ECM) to facilitate cell migration. Recruited cells also secrete a number of growth factors, such as FGF-2, which promote the development of new blood vessels. Many of the fibroblasts take on a myofibroblast phenotype to facilitate wound contraction. **(B)** In order for the wound to close, myofibroblasts deposit and align abundant amounts of ECM, mainly Col-1. The intricate reaction to wounding reduces epithelial adhesiveness and increases epithelial cell mobility to re-form an intact sheet of tissue over the wound. Production of MMPs, uroplasminogen activator (uPA), and tissue plasminogen activator (tPA) facilitates the re-epithelialization. Blood vessels can then enter the fibrin- and fibronectin-rich clot to rapidly vascularize and feed the new tissue. The lateral migration of the epidermal cells is followed by a reversion to their normal state by redepositing basement membrane (BM), reengaging intercellular adhesions, growth arresting, and functionally differentiating. **(C)** Similarly, the tumor microenvironment is populated by immune cells, fibroblasts, and endothelial cells, and tumor cells produce many of the same growth factors that activate the adjacent stromal tissues in wounding in order to create a reactive stroma. **(D)** Tumor cells, cancer-associated fibroblasts (CAFs), and tumor-associated macrophages (TAMs) increase production of MMPs and uPA at the invasive tumor front to stimulate angiogenesis and proliferation. CAFs exert also contractile forces to generate tracks within the ECM through which epithelial cells subsequently invade in a collective fashion.

the vicinity of tumors begin to secrete and deposit factors that cause aberrant growth and invasion? At what stage of progression do epithelial cells (transformed or not) respond? Once a tumor disseminates, what type of microenvironment is necessary for it to grow? And finally, once a reactive stroma has formed, can it be reversed?

Initiation

Perhaps the most convincing demonstration of the microenvironment's influence is that its disruption causes not just aberrant growth but also de novo genetic lesions and full-blown malignancy (Figure 16-4, *A*). This has been shown in mouse models in which ECM remodeling enzymes

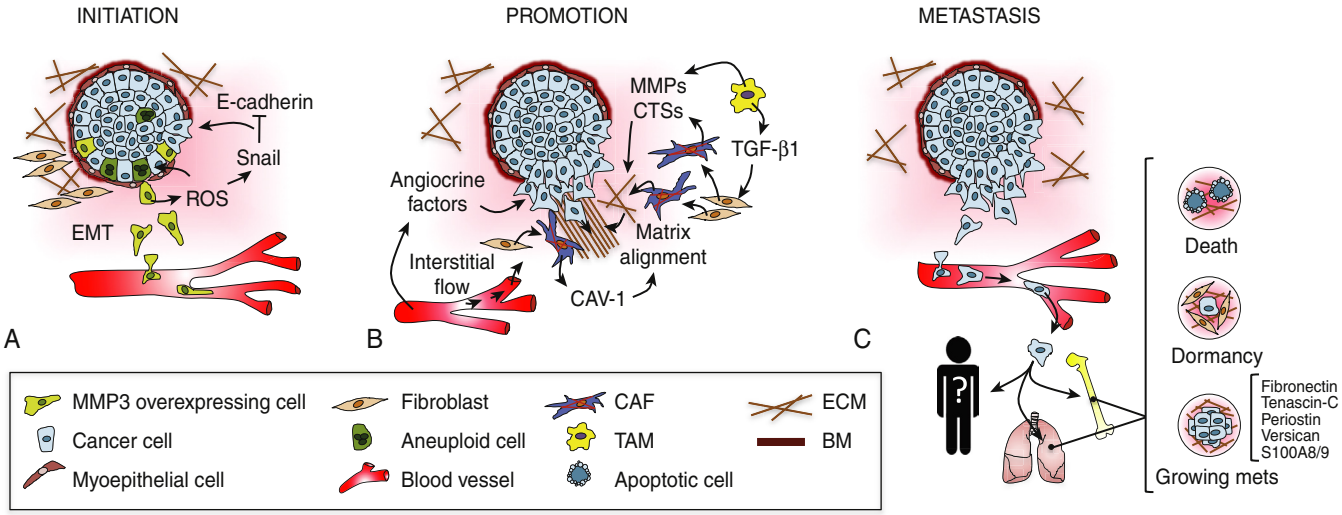


Figure 16-4 The microenvironment is a dominant force in tumor initiation, promotion, and metastasis **(A)** Forcing the expression of a single extracellular matrix (ECM) remodeling enzyme, MMP3, in mammary epithelial cells *in the absence of any additional mutations* initiates genetic lesions and full-blown malignancy. Activation of MMP3 leads to epithelial-to-mesenchymal transitions (EMT), as well as genomic instability through the formation of reactive oxygen species (ROS). In addition, ROS stimulate the expression of Snail, which negatively regulates E-cadherin, loosening cell-cell adhesion and facilitating invasion. **(B)** The microenvironment unleashes initiated epithelium. An increase in the interstitial flow combined with TGF-β1 released by the tumor and by TAMs induces dermal fibroblasts to differentiate into CAFs. These cells secrete several growth factors and cytokines that enhance the proliferation of nearby tumor cells. In addition, CAFs and TAMs produce proteases such as MMPs and cysteine cathepsins (CTSs) to remodel and align the ECM, thus creating tracks through which epithelial cells subsequently invade in a collective fashion. **(C)** The microenvironment is also a potent enhancer of distant metastatic spread. Invasive cancer cells enter local microvasculature and travel through hematogenous and/or lymphatic routes to distant organ sites. Formation of a metastatic niche, characterized by elevated expression of ECM molecules such as fibronectin, tenascin-C, or periostin, may be required in order for disseminated tumor cells to colonize distant tissue.

MMP-3 and MMP-14 are overexpressed in the murine mammary gland.[56,57] Both of these proteases are expressed normally during mammary epithelial morphogenesis and function in part to execute the branching program.[58,59] However, sustained ectopic expression of MMP-3 in luminal epithelial cells (via a MMP-3 transgene driven by a milk protein promoter [WAP], which is primarily active in the murine mammary gland[59]), led to a dramatic upregulation in murine MMP-3 expression in the mammary stroma. This was sufficient to cause the formation of a *reactive stroma* characterized by increased blood vessel density, accumulation of collagen, and expression of ECM molecules typically observed only during development or wounding (e.g., tenascin-C).[60] By 6 months, these mice exhibited substantially more epithelial hyperplasia than their wild-type counterparts, and a small percentage of these mice eventually formed full-blown carcinomas[56] (Figure 16-5, *A*). MMP-3 does not only act on the stroma; epithelial cells are affected directly as well. Exogenous MMP-3 causes oxidative DNA damage through the production of reactive oxygen species (ROS), causing genomic instability and aneuploidy in epithelial cells[61] (see Figure 16-5, *B*). Additionally, ROS stimulate expression of Snail, which negatively regulates E-Cadherin, causing loss of cell-cell adhesion and EMT.[61] In sum, forcing the overexpression of a single ECM remodeling enzyme—without addition of an oncogene or knockdown of a tumor suppressor gene in the mammary gland—results in the manifestation of all of the putative hallmarks of cancer.[55]

More sophisticated transgenic models allow tissue-specific recombination events to facilitate genetic deletion in specific tissue compartments.[62] This strategy has been utilized to demonstrate that introducing a genetic aberration to fibroblasts is sufficient to cause cancer in adjacent epithelium. For instance, driving TGF-β receptor type II (TGF-βRII) deletion by the fibroblast-specific protein 1 (FSP1) promoter (which is expressed ubiquitously by fibroblasts) renders fibroblasts unresponsive to TGF-β signaling and results in a three- to fourfold increase in hepatocyte growth factor (HGF) secretion in the prostate and forestomach of these mice.[63] The ultimate consequence is the induction of proliferative, intraepithelial neoplasms within the prostates of young mice, and of invasive squamous cell carcinomas in the forestomachs of these mice. Similarly, engineering human fibroblasts to overexpress either HGF or TGF-β is sufficient to induce tumorigenic growths from ostensibly normal co-implanted human epithelial tissue.[64]

Fibroblasts also play key roles during tissue development by producing ECM and other molecules that induce growth, branching, and tissue-specific gene expression of resident epithelia. Ostensibly, if fibroblasts were engineered to re-express developmental markers in the mature gland, they have the potential to severely affect epithelial homeostasis. This was tested by co-implanting urogenital sinus

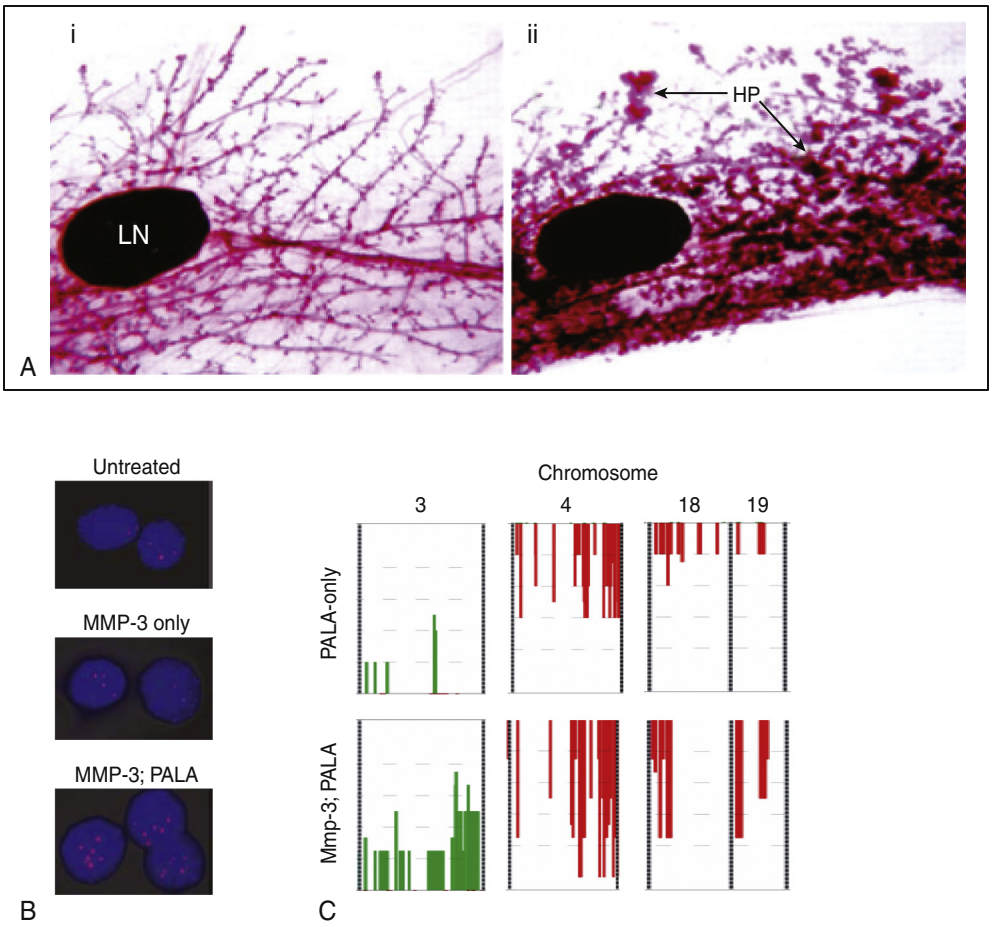


Figure 16-5 Forced overexpression of MMP-3 in the murine mammary gland results in tumor formation in part by causing genomic instability in mammary epithelial cells **(A)** As opposed to nontransgenic control mice (**A** i), WAP-*Mmp3* mice (**A** ii) exhibit multifocal hyperplasia (HP) by 16 months of age. Mmp-3 functions to induce tumorigenesis via reciprocal overexpression of Mmp-3 in the mammary stroma to induce a reactive stroma *(not shown)*, and by acting directly on mammary epithelial cells to induce reactive oxygen species, which cause genomic instability. This is shown by **(B)** amplification of the *CAD* locus *(red)*, which confers resistance to *N*-(phosphonacetyl)-L-aspartate (PALA) treatment, in Mmp-3 treated mammary epithelial cells and by **(C)** comparative genomic hybridization analysis (*green*, amplifications; *red*, deletions) of Mmp-3–treated cells (vs. control [PALA]-treated cells).[56,61] *LN*, lymph node.

mesenchymal cells overexpressing the global epigenetic regulator Hmga2, which is expressed primarily during embryonic development, in a sustained fashion. When implanted with adult prostate cells, Hmga2-overexpressing mesenchyme fostered formation of frank carcinomas primarily by acting on the basal stem cell population of prostate epithelia.[65]

These studies demonstrate that inducing aberrations within the stromal compartment is sufficient, in and of itself, to induce genomic instability, sustained growth, and the transition to an invasive phenotype by uninitiated and untransformed epithelia. Once the epithelium has been initiated, the activated microenvironment also plays a key role in accelerating the progression of transformed epithelium into frank carcinomas.[66,67]

Progression

The previously described wounding studies in RSV-injected chickens[25,30] were among the first to show conclusively that the wounding microenvironment is sufficient to push transformed cells (in this case, those expressing the Src oncogene) to form tumors. More recent works have pinpointed precise roles for specific cell types, growth factors, cytokines, ECM molecules, and associated physicochemical properties in creating a biochemical and mechanical signaling milieu that unleashes initiated/transformed epithelium (see Figure 16-4, *B*).

Before delving into all of the ways that the microenvironment conspires to promote loss of architecture, growth, and invasion, it is important to note that we likely harbor initiated cells throughout our body, which accumulate over the course of our lives as a result of diet, radiation, and so forth.[13,19] Yet on a per-cell level, progression to malignancy is actually an extremely rare event. The reason is that normal tissue architecture supersedes an aberrant genome, as detailed earlier. For epithelial tissues, the BM is the principal biochemical and physical scaffold that must be compromised for in situ disease to become invasive. This specialized, layered ECM

typically consists of at least one member of the laminin family (e.g., both laminin-111 and -332 for mammary epithelium), type IV collagen, nidogens, and heparin sulfate proteoglycans, among others, and signals in a tissue-specific fashion to confer architecture[68] and function to the epithelium.[69,70] Depriving normal cells of BM can have dramatic consequences. Not only does adhesion to BM protect epithelial cells from apoptosis,[71] it confers proper tissue polarity to epithelium. For instance, luminal epithelial cells from the mammary gland "reverse polarize" when removed from BM and cultured in Col-1 (which is found mainly in the stroma)—that is, they express apical proteins basally, and vice versa.[72] Addition of myoepithelial cells, which lie basal to the luminal epithelium and secrete laminin-111 in vivo, restores proper polarity to luminal epithelial cells.[72] Laminin-111 is critical also to tissue-specific gene expression within the mammary gland,[17,73-76] and its organized presence is quite important for preventing carcinoma progression. Co-implantation of myoepithelial cells with a cell line that forms ductal carcinoma in situ–like lesions in vivo restrains progression of these cells even in the presence of CAFs or fibroblasts taken from a chronically inflamed microenvironment.[77] Myoepithelial cells derived from cancer patients, which fail to express laminin-111,[72] cannot confer proper polarity to luminal epithelial cells in culture and fail to prevent fibroblast-mediated invasion of in situ lesions.[77] This establishes a vicious cycle, as loss of epithelial polarity results in upregulated expression of MMP-9, which degrades remaining BM and effects further loss of tissue architecture.[45] Thus, loss of laminins, or inability to produce them, results in loss of tissue architecture, accelerated degradation of preexisting laminin-111, and, if additional necessary signals are present, progression to malignancy. Taken together, these data demonstrate how disruption of tumor-suppressive components within tissues renders cells sensitive to the coercive elements of the tumor microenvironment.

Carcinoma-Associated Fibroblasts and the Factors They Produce Promote Tumor Progression

CAFs account for up to 80% of the fibroblast population in a tumor.[78] They arise from fibroblasts resident to the tissue, as well as vascular smooth muscle cells, pericytes,[79] mesenchymal stem cells,[80] and even epithelial[81] and endothelial cells (via mesenchymal transitions[82]). CAFs phenotypically resemble myofibroblasts—they express fibroblast activation protein, incorporate alpha smooth muscle actin within their actin stress fibers, and deposit copious amounts of fibronectin, including the extra domain (ED)-A–containing splice variant.[49,83] CAF phenotype is stimulated initially, at least in part, by tumor-derived TGF-β1,[84] and the diffusible nature of this stimulation is reflected by the graded pattern of fibroblast activation observed in tumors.[85] Fibroblasts closest to the tumor exhibit the highest level of activation.[79,81,85]

The demonstration that activated stroma associated with tumors actually promotes tumor progression has been known in the literature for decades. An example of increased tumor growth was demonstrated clearly when CAFs isolated from prostate tumors were recombined with SV40-transformed prostate epithelial cells and induced formation of masses more than 10-fold larger than those formed by recombinants composed of normal human prostate fibroblasts and initiated epithelial cells.[86] CAFs from other tissues effect similar outcomes; there is evidence that CAFs play a role in accelerating tumor progression in breast,[87] ovarian,[88] pancreatic,[89] and liver[90] cancers and others. Sustained secretion of growth factors and cytokines such as TGF-β, HGF, SDF-1, and IL-1β by CAFs enhance proliferation and invasion of nearby tumor cells, promote angiogenesis (including recruitment of circulating endothelial progenitor cells), and stimulate a tumor-promoting inflammatory response.[84,87,91,92] However, it should be cautioned that fibroblasts from different organs are not the same, since fibroblasts exhibit substantial heterogeneity between organs.[93] Hence, the mechanisms by which CAFs from a given tissue promote tumor progression are likely to differ as well. Case in point, analysis of CAFs from skin, cervical, mammary, and pancreatic tumors revealed that each have unique expression signatures of a pro-inflammatory gene set.[91]

What is clear from these experiments, which in general require the isolation and expansion of CAFs, is that the CAF phenotype persists in culture despite the absence of a tumor. What is unclear, however, is why. Interestingly, CAFs rarely exhibit somatic genetic alterations[94]; however, on a population level, they do have reduced expression of the well-known tumor suppressors p53 and PTEN. Deletion of PTEN in mammary fibroblasts is sufficient, all on its own, to steer these cells toward a desmoplastic, pro-inflammatory, pro-angiogenic phenotype that drastically accelerates tumor growth in mice.[95] Therefore, epigenetic changes, perhaps caused by sustained overstimulation by TGF-β and SDF-1,[92] or effected even by direct transfer of genetic material from the tumor itself,[96] may drive altered expression profiles in local fibroblasts that are sufficient to induce and sustain the CAF phenotype. Because CAFs persist in culture without the tumor (although the tissue culture plastic milieu is analogous to wounding), the likelihood is that they persist also in vivo once the tumor has been removed. What role residual CAFs play in tumor recurrence has yet to be defined.

CAF-Mediated ECM Remodeling Promotes Loss of Tissue Architecture and Tumor Invasion

In addition to soluble factor–mediated effects, CAFs influence tumor progression by remodeling the ECM in three

distinct but nonexclusive fashions. Destructive remodeling refers to proteolytic breakdown of ECM. Using organotypic skin reconstructs, Gaggioli and colleagues demonstrated that CAFs use a combination of MMP- and force-mediated remodeling of ECM to promote invasion of squamous cell carcinoma (SCC) cells. The authors reached this conclusion after first observing that SCC cells required a physical association with CAFs in order to invade subjacent ECM. Remarkably, preconditioning the underlying ECM with CAFs was still sufficient to induce SCC cell invasion. But how? CAFs secrete MMPs and exert contractile forces to generate tracks within the ECM through which epithelial cells subsequently invade in a collective fashion.[97] Once these tracks are generated, it is quite possible that trailing cells no longer require the proteolytic function of MMP molecules to invade the ECM.[58,98-100] Thus, depending on the amount and type of ECM remodeling that has taken place, inhibiting the proteolytic function of MMPs (which was hailed as a promising strategy for targeting the microenvironment, but has failed to live up to that promise[101]) may prove inadequate to prevent the invasion of tumor cells.

Activated fibroblasts are the principal mediator of desmoplasia or excessive ECM deposition. Tumors exhibit up to 10-fold increases in collagen concentration over physiologic conditions,[102] corresponding to a 24-fold increase in tissue stiffness in a tissue such as the mammary gland.[103] Elevating stiffness out of the physiological range is sufficient to alter the function of normal mammary epithelial cells by altering cell shape,[14] increasing intracellular elasticity, inhibiting tissue-specific gene expression,[104] and causing disorganization of nonmalignant epithelia.[103] Once epithelia are initiated by any means, pathological ECM stiffness drives integrin clustering, focal adhesion formation, ERK activation, and ROCK-mediated contractility, ultimately resulting in disrupted tissue architecture and an invasive phenotype.[103,105,106] Blocking integrin clustering or Rho signaling in 3D culture is sufficient to restore proper epithelial architecture; inhibiting collagen crosslinking in MMTV-Neu mice through lysyl oxidase (LOX) blockade slows tumor progression.[103,105]

Cell-intrinsic and cell-extrinsic forces also have a significant effect on ECM alignment, which in turn profoundly influences tumor invasion. "Tumor-associated collagen signatures" were first described by Provenzano and Keely[107] and refer to three possible alignments of fibrillar collagen observed at the tumor-stroma interface: random, perpendicular/belt-like, or radially aligned. The last is observed at invasive tumor fronts, and patients with radially aligned collagen at the tumor-stroma interface have significantly diminished disease-free and overall survival.[109-110] One potential mediator of collagen alignment is the tumor itself, particularly in instances where stiffening of the stroma enhances contractility of tumor cells.[105,111] Physical stimulus from the microenvironment also triggers ECM alignment by fibroblasts. Hydrostatic pressure drives water out of capillaries into the interstitial (tissue) space to be collected, in part, by lymphatic vessels present within the tissue. This interstitial flow can increase by an order of magnitude in a tumor.[112] When subjected to pathological flow rates, dermal fibroblasts differentiate into myofibroblasts and align themselves and their surrounding ECM perpendicular to the direction of flow.[113] Strain from the aligned ECM may potentiate TGF-β1 activation by allowing fibroblasts to physically pull apart the molecule from its ECM-bound latent complex,[114] creating a feed-forward loop to sustain the activated phenotype. The aligned ECM, in conjunction with factors secreted by activated fibroblasts, can then direct tumor invasion.

Recently, a molecular mediator of CAF-mediated ECM alignment was uncovered. This molecule, known as Caveolin-1 (Cav-1), is a scaffolding protein essential to the structure of caveolae, or "little caves," in cellular plasma membranes.[114] Cav-1 assists with focal adhesion maturation[115] and promotes force-dependent remodeling of surrounding ECM[116] by embryonic fibroblasts. Although loss of Cav-1 in patient stroma is associated with increased primary tumor size,[117,118] Cav-1–mediated ECM remodeling by fibroblasts enhances invasion and distant metastatic spread. In coculture assays consisting of breast tumor cells and embryonic fibroblasts, Cav-1 expression within the fibroblast compartment potentiates directional alignment of Col-1 and fibronectin-rich ECM to facilitate tumor cell invasion.[116] *Cav-1*–null fibroblasts are unable to align ECM in culture and fail to promote distant metastasis of co-implanted breast cancer cells in vivo.[116] These findings open the door for therapies that target both CAF-derived soluble factors that promote tumor growth, and molecules such as Cav-1 that promote CAF-mediated disruption of tissue architecture to facilitate tumor invasion. However, although CAFs are representative of how nontumor cells function in the tumor microenvironment, they are only part of the story. It is important to realize that other cell types can also be induced to aid tumor cell survival, growth, and invasion.

Other Cell Types Contribute to the Tumor Microenvironment

Our bodies consist of more than 300 different cell types, a subset of which constantly engage each other in any given organ to direct development and maintain homeostasis. One would expect that few, if any, of the cells in an organ act as silent bystanders during tumor initiation, formation, and progression. Thus, although we describe known roles for immune cells and endothelial cells later, the reader should not infer that any cell types not mentioned are uninvolved. Instead, the reader should consider these unknown interactions as potential avenues of exploration and an opportunity to define new connections that shape the tumor microenvironment.

Because the immune system is known to protect the host, it was expected that immune cells would be protected against cancer. However, it is now clear that a subset of immune cells in fact promote tumor progression. No immune cell type embodies this paradigm shift better than the macrophage, which was long pigeonholed as a phagocytic cell tasked with rejecting a tumor until its trophic functions in development and disease became clear.[119,120] Macrophages have been classified based on their mode of activation: classically activated/M1 macrophages respond to interferon-γ by releasing pro-inflammatory cytokines and are involved in T helper 1 cell–mediated resolution of acute infection, whereas alternatively activated/M2 macrophages respond to cytokines from T helper 2 cells and are involved in wounding and fibrosis.[119] To some, this classification is overly restrictive and ignores the phenotypic diversity displayed by macrophages, given that they also maintain bone homeostasis,[121] promote ductal branching or involution of the mammary gland,[122,123] function in various steps of the angiogenic cascade,[119] and guide neural networking.[124] These diverse functions are executed in a tissue- and context-specific fashion by a number of discrete macrophage subtypes, which aid these developmental processes by remodeling collagen and secreting a host of pro-angiogenic, pro-inflammatory, and matrix-degrading factors (reviewed in Ref. 125).

Tumor-associated macrophages (TAMs) are M2-like in function, and their presence correlates with increased vascular density and poor clinical outcome for a number of human cancers, including breast, lung, and ovarian cancer.[119] A principal mediator of macrophage recruitment to the tumor microenvironment is colony stimulating factor (CSF)-1. Once there, CD4-positive ($CD4^+$) and $CD8^+$ T cells steer recruited macrophages toward an M2-like phenotype via IL-4. Diminishing CSF-1 levels[126] or neutralizing IL-4[127] have similar effects on mammary tumor progression. Whereas neither of these manipulations inhibits tumor growth, tumor progression is slowed, and mice have significantly fewer metastases.[126,127] TAMs promote progression and metastasis through ECM remodeling and by secreting many of the same trophic factors released by M2-like macrophages during development, such as extracellular proteases that degrade BM, disrupt epithelial architecture, and enable invasion[126,128]; angiogenic factors that promote new blood vessel formation[129,130]; and epidermal growth factor (EGF) as part of a chemotactic EGF-CSF1 paracrine loop that mediates co-invasion of tumor cells and macrophages.[127,131]

It is important to note that immune cell distributions are tissue-specific beyond macrophage subsets and involve a host of other leukocytes in normal organs and during tumor progression.[132] Thus, in order to design therapies that effectively target tumor-associated inflammation, it is likely as important to understand how leukocyte populations shift during tumor progression as it is to understand how different leukocytes change phenotypic characteristics.

Blood vessels are generally regarded as conduits for oxygen, nutrients, and hematopoietic cells and thus as passive participants in the tumor microenvironment.[133] However, this is not the case. Endothelial cells are active participants in the dynamic interactions that occur between cells in any tissue. This was established first in development: endothelial cells secrete soluble factors that stimulate liver growth and tissue-specific gene expression from the pancreas in developing embryos.[134,135] More recently, endothelial cell–derived "angiocrine factors" have been shown to comprise niches that maintain stem cells in both brain[136,137] and bone marrow.[138-140] Increasing evidence suggests also that NOTCH ligands and specific BM molecules expressed in the brain perivasculature mediate the survival of glioma-initiating cells and disseminated tumor cells in the brain.[141-144]

Significant tumor-promoting roles have also been defined for other immune cells,[145] as well as endothelial progenitor cells,[146] mesenchymal stem cells,[80,147] neurons,[148] and adipocytes.[149] We direct the reader to the references listed to learn more about the contributions that these cell types make to the tumor microenvironment.

Metastasis

The study of the microenvironment's role in metastasis returns us to the beginning of the chapter and harks back to Paget's observation regarding the spread of breast cancer that was the basis of his "seed and soil" hypothesis.[18] The mechanistic underpinnings of this hypothesis are perhaps the hottest topic in metastasis research today.[150] Whereas it has been postulated that tumor cells actively "home" to a given organ site, there is also evidence that tumor cells spread indiscriminately.[151] In this latter case, certain secondary tissue microenvironments, or "soils," must be particularly hospitable for growth of disseminated tumor cells (DTCs). This could happen in three ways: (1) tumor cells preferentially remodel target organ sites *before* they get there (i.e., they form a premetastatic niche); (2) tumor cells bring their own microenvironment with them; or (3) surviving DTCs are dependent on formation of a niche that favors their growth after arriving to the secondary site. The dynamics of metastatic relapse displayed by cancer patients suggest that all three of these mechanisms are plausible,[152] and experimental data from spontaneous and experimental metastasis assays in mice offer some insight. We discuss these three possibilities in more detail next.

The concept of the premetastatic niche refers to the priming of distant organs by tumor-derived factors. This was demonstrated first in immune-competent mice inoculated subcutaneously with either B16 melanoma or Lewis lung carcinoma cells. In these mice, $VEGFR1^+$ bone marrow–derived

progenitor cells (BMDCs) are recruited to future sites of metastasis before even the first tumor cells arrive.[153] On arriving at target organs, BMDCs secrete the chemokine SDF-1 to recruit tumor cells,[154] upregulate fibronectin expression in these tissues to promote engraftment and growth of the recruited tumor cells, and activate MMP-9 to destroy BM, disorganize epithelial tissues, and liberate VEGF from the ECM.[45,155] In addition to promoting angiogenesis within the tissue, VEGF functions to enhance permeability of the microvasculature and to recruit $VEGFR2^+$ BMDCs that contribute to new blood vessel formation. The ultimate result is more rapid activation of the angiogenic switch and hence accelerated metastatic outgrowth.[153] Subsequent studies have pinpointed induction of MMP-2, S100A8, and S100A9 at secondary sites as other principal constituents of the premetastatic niche.[156,157]

Tumor-derived agents that induce formation of the premetastatic niche continue to be uncovered. In a study by Kaplan and colleagues, media conditioned by B16 melanoma cells, which metastasize to the lung, liver, testis, spleen, and kidney, primed Lewis lung carcinoma cells, which metastasize only to the lung and liver, to metastasize to a wider array of organs.[153] What is it in the tumor-conditioned medium that causes this effect? Whereas tumor-derived factors such as VEGF-A, VEGF-C, and LOX have been implicated in modulating distant microenvironments,[156,158,159] secretion of soluble factors, which ostensibly circulate systemically and thus have the potential to modulate any organ site, does not account for the organ specificity of premetastatic niche formation.

A breakthrough study revealed that tumors can execute tissue-specific remodeling of distant microenvironments through *exosomes*, small (30 to 100 nm) cell membrane–derived microvesicles packed with a selected number of molecules. Cargo carried by exosomes includes cytokines, growth factors, ECM proteins, mRNA, microRNAs, and even phosphorylated signaling proteins.[160] The exciting discovery that exosomes can be transferred from cell to cell would indicate that tumor-derived exosomes can activate or deactivate a number of signaling pathways in recipient cells to effect tissue remodeling from near or far.[160]

Specific exosome cargos evolve with tumor progression. For instance, a number of proteins are upregulated in exosomes from metastatic melanoma (compared to nonmetastatic disease), including the oncoprotein MET.[161] Preconditioning mice with exosomes from highly metastatic melanoma cells causes subsequently injected poorly metastatic melanoma cells to home to a greater number of organs and rapidly form lethal metastases.[161] How? Melanoma exosomes home to target organs (possibly influenced by expression of specific integrins on their surface) and induce vascular permeability to promote entry of circulating tumor cells. In addition, exosomes are taken up by BMDCs, resulting in a MET-directed education toward a pro-vasculogenic phenotype. Pro-vasculogenic BMDCs promote metastatic outgrowth of disseminated cells on arriving to target organs.[161] Accordingly, exosomes derived from metastatic tumor cells can prime distant organ sites for tumor cell extravasation, adhesion, and growth. Thus inhibiting exosome production, engraftment, or specific exosome contents could prove effective in blocking or disrupting formation of the pre-metastatic niche.

Whereas tumor cells and tumor-derived exosomes have been observed in the blood, only recently has the possibility been raised that stromal cells from the tumor microenvironment may also wind up in the body's circulation. Is it possible that tumor cells could be bringing their own soil to distant organs? A small-scale study revealed that nonepithelial, nonlymphocytic, fibroblast-like cells were present in the blood of more than 50% of patients with metastatic disease, but absent in all participants with localized cancer.[162] Experiments in mice have shown the potential significance of circulating fibroblasts, as tumor cells in heterotypic tumor-fibroblast fragments exhibit enhanced survival and enhanced efficiency of metastatic outgrowth on reaching target organs.[163,164]

Aside from preconditioning metastatic sites before their arrival or bringing activated stromal cells with them as they metastasize, tumor cells are also able to disrupt homeostasis in target organs and induce stromal cells to deposit factors that create a favorable *metastatic niche*[165] (see Figure 16-4, *C*). Metastatic tumor cell lines can be enriched for lung, bone, and brain tropism by serial passaging through mice, and these cells reveal distinct gene expression signatures that enable more efficient metastasis to these target organs.[166-168] The products of a number of these genes mediate interactions between the tumor and its microenvironment. Follow-up studies have defined a number of ECM molecules and extracellular factors such as tenascin-C,[169] periostin,[170] versican,[171] col-1,[172] interleukin-6,[173] and tissue factor[174] that make up the metastatic niche. From these studies, two themes emerge: (1) it is not necessary for both the tumor and the stromal cells to express these factors—either will do, and induction could be reciprocal; and (2) these molecules are also commonly expressed during development or wounding in a temporally regulated fashion. As a result, the metastatic niche comprises a familiar, hospitable milieu that supports DTC survival and growth.

The Frontiers of the Microenvironment

There are a number of conclusions that can be made from the newly appreciated field of the microenvironment. One is that the microenvironment can have both positive and negative influences on aberrant cells. The second is that the normal microenvironment may indeed protect against tumor

progression (for review, see Ref. 13). In addition, two of the most crucial directions in this research are understanding how the microenvironment contributes to chemotherapeutic resistance, and how therapeutic regimens could be devised that simultaneously target tumor cells and their surrounding microenvironment.

The Tumor Microenvironment Promotes Drug Resistance

A popular theory about how tumors become chemoresistant is that a given chemotherapeutic kills all of the susceptible cells. The fraction that survives then clonally expands, and the genetic alterations that rendered these cells resistant largely remain, resulting in a tumor that may no longer respond to that given therapy.[175] However, it is increasingly appreciated that the microenvironment also significantly affects treatment response.

One way it does so is by influencing the delivery and distribution of administered drugs. Blood vessels in tumors are highly heterogeneous in terms of blood flow and permeability.[176,177] Thus, depending on its molecular weight, a drug may be able to enter one portion of a tumor, but never reach another.[176] Even if it exits the vasculature, drug transport through the bulk of the tumor is greatly influenced by the density of cells and the ECM molecules. Cell density affects the effective concentration of drug taken up by tumor cells, whereas ECM molecules restrict the effective diffusion of a drug via binding interactions and/or by physically restricting mobility through the proteinaceous mesh.[69,176,178] Thus, one may conclude that pretargeting fibrosis and desmoplasia is a promising approach to improve the distribution and efficacy of subsequently delivered chemotherapeutics. Indeed, initial attempts at this approach by pretreating tumors with ECM-degrading or antifibrotic agents before administering chemotherapeutics appear quite promising.[179,180]

Aside from directly affecting delivery of drugs, it is apparent also that privileged niches within the microenvironment protect tumor cells from the damaging effects of therapeutics. One such niche is the BM. It has long been established that β1 integrin–mediated binding of BM suppresses apoptosis of normal mammary epithelial cells.[71,181] Binding of a principal constituent of BM—laminin-111—via integrin-β1 has also been shown to mediate resistance of small-cell lung carcinoma to cytotoxic agents.[182] BM also directs formation of polarized acini that render mammary epithelial cells resistant to a host of cytotoxic drugs and death receptor ligands independent of growth status. This laminin-induced resistance to apoptosis is transmitted via integrin $\alpha_6\beta_4$ through formation of mature hemidesmosomes to direct cytoskeletal organization and polarity.[183]

Adhesion to other ECM molecules also plays a role in therapeutic resistance. For instance, engagement of the fibronectin ED-A splice variant via integrin $\alpha_5\beta_1$ diminishes the radioresponse of breast cancer cells,[184] and association with type VI collagen renders ovarian cancer cells resistant to cisplatin.[185] These molecules as well as others are likely to act by hyperactivating integrin-mediated survival pathways (e.g., integrin→focal adhesion kinase→Akt),[186] thus providing tumor cells with a survival advantage.

It is also important to consider the effects of chemotherapeutics on nontumor cells, and how their response alters the tumor microenvironment. For instance, normal epithelial cells and stromal fibroblasts respond to the chemotherapeutic mitoxantrone by shifting toward a senescence-associated secretory phenotype. This phenotype is characterized by secretion of a host of molecules associated with angiogenesis, immune cell recruitment, and EMT.[187] Hence, a possible by-product of treatment is that the reactive stroma secretes factors that stimulate nearby tumor cells to grow, invade, and spread. This applies not just to resident tissue cells, but to recruited myeloid cells as well.[188-190]

Because tumor cells are able to successfully seek refuge in a variety of protective niches, single therapies are highly unlikely to retain their efficacy over the long-term. An attractive solution is to devise therapeutic regimens that target the tumor and its microenvironment simultaneously or in stepwise fashion to deprive the tumor of the interactions that sustain it in the face of chemotherapy.

Targeting the Tumor Microenvironment

In this chapter, we have provided a number of examples of how reciprocal interactions between cells and their microenvironment function to initiate tumors, force progression, facilitate metastatic outgrowth, and compose a privileged niche that confers therapeutic resistance. A logical extension of these findings is that inhibiting critical interactions between tumor cells and their microenvironmental constituents could slow tumor progression and render these cells more susceptible to treatment. The growing literature on the importance of microenvironment that now spans more than a century offers a blueprint as to how this can be accomplished, including:

1. Targeting reactive elements of the tumor microenvironment. This notion has spurred a number of studies aimed at testing new therapies (and revisiting others) that target angiogenesis, fibrosis, inflammation, desmoplasia, and other aspects of the tumor microenvironment. A brief summary of these drugs is presented in Table 16-1 (for a detailed list, see Ref. 13).

Table 16-1 A Sampling of Drugs Developed to Target the Tumor Microenvironment

Common Name (Trade Name)	Company	Drug Information
Bevacizumab (Avastin)	Genentech/Roche	In 2004, bevacizumab became the first FDA-approved angiogenesis inhibitor. It is a humanized monoclonal antibody with specific affinity for VEGF-A, thus inhibiting the signaling between the tumor and endothelial cells in the microenvironment.[191]
Sunitinib, SU11248 (Sutent)	Pfizer	Sunitinib garnered FDA approval in 2006. A small-molecule receptor tyrosine kinase inhibitor, it inhibits signaling through PDGFRα, PDGFRβ, VEGFR1, VEGFR2, VEGFR3, KIT, FLT3, CSF-1R, and RET to disrupt angiogenesis, macrophage recruitment, lymphangiogenesis, and tumor cell growth.
Endostatin (Endostar)	Simcere	Endostatin is a 20-kDa peptide fragment derived from the extracellular matrix protein collagen XVIII.[192] Endostatin has a potent effect on endothelial cell proliferation and angiogenesis. It is currently used therapeutically (and in clinical studies) only in China under the trade name Endostar.
Sorafenib, BAY 43-9006 (Nexavar)	Bayer	Approved by the FDA in 2005, sorafenib is a small-molecule kinase inhibitor that inhibits many intracellular and extracellular kinases. Most affected are Raf kinase, VEGF receptor, and PDGFRs, thus resulting in reduced tumor growth and angiogenesis.[193]
MK-2461	Merck	MK-2461 is a small-molecule inhibitor of c-MET kinase, the receptor of the stromal-derived hepatocyte growth factor (HGF). c-MET activation stimulates proliferation and invasion of tumor cells and also stimulates angiogenesis.[194]
Zoledronate (Zometa)	Novartis	Zoledronate belongs to the bisphosphonate class of drugs. It inhibits bone resorption by osteoclasts. Also inhibited is the differentiation of myeloid cells; TAMs are also affected.[195,196]
Denosumab (Xgeva)	Amgen	Approved by the FDA in 2010, denosumab is a human antibody that binds to human receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL regulates osteoclastogenesis and is involved in pathways regulating osteoclastogenesis and tumor cell metastasis to bone, as well as endothelial cell proliferation and apoptosis.[197,198]
Anastrozole (Armidex)	Novartis	Anastrozole is a third-generation inhibitor of aromatase, a cytochrome p450 complex present in the stromal fibroblasts and adipocytes. Aromatase catalyzes the conversion of androgens to estrogens; the inhibitors are approved for the treatment of breast cancer in postmenopausal women.
AMD070	Genzyme	Currently in clinical trials, AMD070 belongs to a class of drugs that inhibit CXCR4. CXCR4 is specific for stromal-derived factor 1 (SDF-1/CXCL12) ligand, which is predominantly expressed by fibroblasts and pericytes.
DX2400	Dyax	In development, DX2400 is a new-generation MMP inhibitor, a human monoclonal antibody specific for MMP14. Previous broad-spectrum MMP inhibitors were generally plagued by a lack of efficacy. However, since the end of the previous trials, much has been learned about MMPs, notably the need for specificity. Some are regarded as being protective, and others not. Thus, newer inhibitors are being designed with specificity in mind.[199]
MK0822	Merck	MK0822 is an inhibitor of cathepsin K, a secreted protease involved in bone resorption. Similar to bisphosphonates, inhibitors of cathepsin K proteases may protect against bone loss induced by metastatic tumor cells.[200] Cathepsin inhibitors may be useful also in blocking cathepsin-mediated protumor effects of TAMs.[201]
TGF-β2 AP12009 (Trabedersen)	Antisense Pharma	Trabedersen is an antisense oligodeoxynucleotide with specificity toward TGFβ2. It is currently in phase I, II, and III clinical trials and is being developed for the treatment of tumors that exhibit high levels of TGFβ2 (pancreatic carcinoma, melanoma, and gliomas). Reductions in TGFβ2 in the tumor are likely to be profound, affecting both tumor and stromal cells (tumor cell growth, angiogenesis, and immune response).
AVE1642	Immunogen/Sanofi Aventis	AVE1642, a humanized monoclonal antibody, is a specific antagonist of the insulin-like growth factor 1 receptor (IGF-1R). IGF-1 derived from bone-marrow stroma promotes survival and growth of multiple myeloma cells. IGF-1R signaling also contributes to angiogenesis via its influence on HIF1α/VEGF expression.[202]
BGJ398	Novartis	Currently in clinical trials, BGJ398 is a small-molecule inhibitor of fibroblast growth factor receptors (FGFR). The ligands of this receptor, FGFs, are expressed by the activated fibroblasts of tumor stroma and have a protumorigenic effect.
Bortezomib, PS-341 (Velcade)	Millennium Pharmaceuticals	Bortezomib is a novel inhibitor of the 26S proteasome complex and is indicated for the treatment of relapsed multiple myeloma and mantle-cell lymphoma. In addition to direct inhibition of the tumor cells, bortezomib interferes with MM tumor and bone marrow stromal cell interactions, inhibiting cytokine signaling and angiogenesis.[203]
PEGPH20	Halozyme	PEGPH20 is a covalently modified form of hyaluronidase, which catalyzes the degradation of the extracellular matrix component hyaluronan. In preclinical animal models, PEGPH20 led to dramatic reductions of the tumor interstitial fluid pressure, subsequently enhancing the delivery of co-administered drugs.[179] PEGPH20 is currently in Phase II clinical trials for patients with stage IV pancreatic cancer.[204]

2. Inhibiting signals that impart chemotherapeutic resistance, e.g., β1-integrin–mediated signaling, prior to or coincident with administration of chemotherapeutics or radiation.
3. Reverting the tumor microenvironment. A more complete understanding about the signals that end the development of tissues, or those that deactivate wounding stroma, could lead to therapies based on these same cues that neutralize tumor stroma.

In the end, it is important to remember that the tumor and its microenvironment do not function in a vacuum. They are constantly engaged in dynamic and reciprocal interactions, and it is the balance of signaling among all of these components that is key to homeostasis. Disrupting this balance without correction can result in tumor initiation and accelerated progression. Remember also that there is likely no uniform way to restore the balance of signaling in a tumor. Just as tumor types are different, tumor microenvironments differ from tumor to tumor and from patient to patient. Accordingly, one should investigate multiple therapeutic avenues and remain open to how these can be applied in concert, as this is likely to provide the best means to manage tumors and improve patient outcome.

Acknowledgements

The authors are grateful to Ms. Negest Williams for unparalleled administrative support.

Key References

2. Check Hayden E. Human genome at ten: life is complicated. *Nature*. 2010;464:664-667.
3. Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature*. 2012;486:405-409.
4. Koboldt DC, Fulton RS, McLellan MD, et al. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
9. Weigelt B, Glas AM, Wessels LF, Witteveen AT, Peterse JL, van't Veer LJ. Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci U S A*. 2003;100:15901-15905.
10. Schmidt-Kittler O, Ragg T, Daskalakis A, et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proc Natl Acad Sci U S A*. 2003;100:7737-7742.
11. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. 2008;13:58-68.
12. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science*. 2008;321:1841-1844.
13. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med*. 2011;17:320-329.
19. Folkman J, Kalluri R. Cancer without disease. *Nature*. 2004;427:787.
40. Lee LM, Seftor EA, Bonde G, Cornell RA, Hendrix MJ. The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation. *Dev Dyn*. 2005;233:1560-1570.
42. Sandal T, Valyi-Nagy K, Spencer VA, Folberg R, Bissell MJ, Maniotis AJ. Epigenetic reversion of breast carcinoma phenotype is accompanied by changes in DNA sequestration as measured by AluI restriction enzyme. *Am J Pathol*. 2007;170:1739-1749.
45. Beliveau A, Mott JD, Lo A, et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. *Genes Dev*. 2010;24:2800-2811.
46. Bissell MJ, Kenny PA, Radisky DC. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. *Cold Spring Harb Symp Quant Biol*. 2005;70:343-356.
52. Li B, Wang JH. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability*. 2011;20:108-120.
53. Jain RK. Molecular regulation of vessel maturation. *Nat Med*. 2003;9:685-693.
58. Mori H, Lo AT, Inman JL, et al. Transmembrane/cytoplasmic, rather than catalytic, domains of Mmp14 signal to MAPK activation and mammary branching morphogenesis via binding to integrin beta1. *Development*. 2013;140:343-352.
61. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*. 2005;436:123-127.
63. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*. 2004;303:848-851.
64. Kuperwasser C, Chavarria T, Wu M, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci U S A*. 2004;101:4966-4971.
65. Zong Y, Huang J, Sankarasharma D, et al. Stromal epigenetic dysregulation is sufficient to initiate mouse prostate cancer via paracrine Wnt signaling. *Proc Natl Acad Sci U S A*. 2012;109:E3395-E3404.
67. Monnier Y, Farmer P, Bieler G, et al. CYR61 and alphaVbeta5 integrin cooperate to promote invasion and metastasis of tumors growing in preirradiated stroma. *Cancer Res*. 2008;68:7323-7331.
68. Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol*. 2011;3:2-27.
69. Ghajar CM, Bissell MJ. Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. *Histochem Cell Biol*. 2008;130:1105-1118.
73. Xu R, Nelson CM, Muschler JL, Veiseh M, Vonderhaar BK, Bissell MJ. Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. *J Cell Biol*. 2009;184:57-66.
77. Hu M, Yao J, Carroll DK, et al. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*. 2008;13:394-406.
80. Spaeth EL, Dembinski JL, Sasser AK, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One*. 2009;4: e4992.
81. Petersen OW, Nielsen HL, Gudjonsson T, et al. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol*. 2003;162:391-402.
82. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res*. 2007;67:10123-10128.
83. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6:392-401.
85. Gao MQ, Kim BG, Kang S, et al. Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial-mesenchymal transition-like state in breast cancer cells in vitro. *J Cell Sci*. 2010;123:3507-3514.
87. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.

88. Schauer IG, Sood AK, Mok S, Liu J. Cancer-associated fibroblasts and their putative role in potentiating the initiation and development of epithelial ovarian cancer. *Neoplasia*. 2011;13:393-405.
89. Bachem MG, Schunemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128:907-921.
90. Mazzocca A, Fransvea E, Dituri F, Lupo L, Antonaci S, Giannelli G. Down-regulation of connective tissue growth factor by inhibition of transforming growth factor beta blocks the tumor-stroma cross-talk and tumor progression in hepatocellular carcinoma. *Hepatology*. 2010;51:523-534.
91. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell*. 2010;17:135-147.
92. Kojima Y, Acar A, Eaton EN, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A*. 2010;107:20009-20014.
94. Qiu W, Hu M, Sridhar A, et al. No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. *Nat Genet*. 2008;40:650-655.
95. Trimboli AJ, Cantemir-Stone CZ, Li F, et al. Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*. 2009;461:1084-1091.
96. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res*. 2010;70:9621-9630.
97. Gaggioli C, Hooper S, Hidalgo-Carcedo C, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol*. 2007;9:1392-1400.
98. Fisher KE, Sacharidou A, Stratman AN, et al. MT1-MMP- and Cdc42-dependent signaling co-regulate cell invasion and tunnel formation in 3D collagen matrices. *J Cell Sci*. 2009;122:4558-4569.
99. Wolf K, Mazo I, Leung H, et al. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol*. 2003;160:267-277.
100. Correia AL, Mori H, Chen EI, Schmitt FC, Bissell MJ. The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90beta. *Genes Dev*. 2013;27:805-817.
101. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer*. 2006;6:227-239.
103. Paszek MJ, Zahir N, Johnson KR, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell*. 2005;8:241-254.
104. Alcaraz J, Xu R, Mori H, et al. Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. *EMBO J*. 2008;27:2829-2838.
105. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009;139:891-906.
106. Wozniak MA, Desai R, Solski PA, Der CJ, Keely PJ. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol*. 2003;163:583-595.
107. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med*. 2006;4:38.
108. Conklin MW, Eickhoff JC, Riching KM, et al. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol*. 2011;178:1221-1232.
111. Provenzano PP, Inman DR, Eliceiri KW, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med*. 2008;6:11.
113. Ng CP, Hinz B, Swartz MA. Interstitial fluid flow induces myofibroblast differentiation and collagen alignment in vitro. *J Cell Sci*. 2005;118:4731-4739.
114. Wipff PJ, Rifkin DB, Meister JJ, Hinz B. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol*. 2007;179:1311-1323.
115. Goetz JG, Joshi B, Lajoie P, et al. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. *J Cell Biol*. 2008;180:1261-1275.
116. Goetz JG, Minguet S, Navarro-Lerida I, et al. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell*. 2011;146:148-163.
117. Sloan EK, Ciocca DR, Pouliot N, et al. Stromal cell expression of caveolin-1 predicts outcome in breast cancer. *Am J Pathol*. 2009;174:2035-2043.
118. Witkiewicz AK, Dasgupta A, Sotgia F, et al. An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. *Am J Pathol*. 2009;174:2023-2034.
119. Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol*. 2009;9:259-270.
120. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 2004;4:71-78.
122. O'Brien J, Martinson H, Durand-Rougely C, Schedin P. Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development*. 2012;139:269-275.
123. Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn*. 2006;235:3222-3229.
125. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141:39-51.
127. DeNardo DG, Barreto JB, Andreu P, et al. $CD4^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*. 2009;16:91-102.
128. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
129. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res*. 2007;67:5064-5066.
130. Lin EY, Li JF, Gnatovskiy L, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*. 2006;66:11238-11246.
131. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 2004;64:7022-7029.
132. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science*. 2013;339:286-291.
136. Mathieu C, Sii-Felice K, Fouchet P, et al. Endothelial cell-derived bone morphogenetic proteins control proliferation of neural stem/progenitor cells. *Mol Cell Neurosci*. 2008;38:569-577.
137. Shen Q, Goderie SK, Jin L, et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science*. 2004;304:1338-1340.
143. Zhu TS, Costello MA, Talsma CE, et al. Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer Res*. 2011;71:6061-6072.
144. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. 2007;11:69-82.
145. Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. *Genes Dev*. 2011;25:2559-2572.
147. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
148. Campbell JP, Karolak MR, Ma Y, et al. Stimulation of host bone marrow stromal cells by sympathetic nerves promotes breast cancer bone metastasis in mice. *PLoS Biol*. 2012;10: e1001363.
149. Chandler EM, Seo BR, Califano JP, et al. Implanted adipose progenitor cells as physicochemical regulators of breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:9786-9791.
150. Lyden D, Welch DR, Psaila B, eds. *Cancer Metastasis: Biologic Basis and Therapeutics*. New York, NY: Cambridge University Press; 2011.
151. Suzuki M, Mose ES, Montel V, Tarin D. Dormant cancer cells retrieved from metastasis-free organs regain tumorigenic and metastatic potency. *Am J Pathol*. 2006;169:673-681.
152. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7:834-846.
153. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-827.
156. Erler JT, Bennewith KL, Cox TR, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*. 2009;15:35-44.

157. Hiratsuka S, Watanabe A, Sakurai Y, et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol*. 2008;10:1349-1355.
158. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood*. 2007;109:1010-1017.
159. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med*. 2005;201:1089-1099.
160. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol*. 2011;21:139-146.
161. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18:883-891.
162. Jones ML, Siddiqui J, Pienta KJ, Getzenberg RH. Circulating fibroblast-like cells in men with metastatic prostate cancer. *Prostate*. 2013;73:176-181.
163. Duda DG, Duyverman AM, Kohno M, et al. Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci U S A*. 2010;107:21677-21682.
164. Xu Z, Vonlaufen A, Phillips PA, et al. Role of pancreatic stellate cells in pancreatic cancer metastasis. *Am J Pathol*. 2010;177:2585-2596.
165. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. 2009;9:285-293.
166. Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. *Nature*. 2009;459:1005-1009.
167. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;436:518-524.
168. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 2003;3:537-549.
169. Oskarsson T, Acharyya S, Zhang XH, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. 2011;17:867-874.
170. Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2012;481:85-89.
171. Kim S, Takahashi H, Lin WW, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature*. 2009;457:102-106.
172. Barkan D, El Touny LH, Michalowski AM, et al. Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. *Cancer Res*. 2010;70:5706-5716.
173. Studebaker AW, Storci G, Werbeck JL, et al. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res*. 2008;68:9087-9095.
174. Gil-Bernabe AM, Ferjancic S, Tlalka M, et al. Recruitment of monocytes/macrophages by tissue factor-mediated coagulation is essential for metastatic cell survival and premetastatic niche establishment in mice. *Blood*. 2012;119:3164-3175.
175. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004;10:789-799.
177. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. 2005;15:102-111.
179. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012;21:418-429.
180. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A*. 2011;108:2909-2914.
184. Nam JM, Onodera Y, Bissell MJ, Park CC. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. *Cancer Res*. 2010;70:5238-5248.
185. Sherman-Baust CA, Weeraratna AT, Rangel LB, et al. Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. *Cancer Cell*. 2003;3:377-386.
186. Serebriiskii I, Castello-Cros R, Lamb A, Golemis EA, Cukierman E. Fibroblast-derived 3D matrix differentially regulates the growth and drug-responsiveness of human cancer cells. *Matrix Biol*. 2008;27:573-585.
187. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. 2008;6:2853-2868.
188. Acharyya S, Oskarsson T, Vanharanta S, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell*. 2012;150:165-178.
189. Nakasone ES, Askautrud HA, Kees T, et al. Imaging tumor-stroma interactions during chemotherapy reveals contributions of the microenvironment to resistance. *Cancer Cell*. 2012;21:488-503.
190. Shree T, Olson OC, Elie BT, et al. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev*. 2011;25:2465-2479.
191. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov*. 2004;3:391-400.
192. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88:277-285.
193. Kupsch P, Henning BF, Passarge K, et al. Results of a phase I trial of sorafenib (BAY 43-9006) in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer. *Clin Colorectal Cancer*. 2005;5:188-196.
194. Pan BS, Chan GK, Chenard M, et al. MK-2461, a novel multitargeted kinase inhibitor, preferentially inhibits the activated c-Met receptor. *Cancer Res*. 2010;70:1524-1533.
195. Wolf AM, Rumpold H, Tilg H, Gastl G, Gunsilius E, Wolf D. The effect of zoledronic acid on the function and differentiation of myeloid cells. *Haematologica*. 2006;91:1165-1171.
196. Veltman JD, Lambers ME, van Nimwegen M, et al. Zoledronic acid impairs myeloid differentiation to tumour-associated macrophages in mesothelioma. *Br J Cancer*. 2010;103:629-641.
197. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289:1504-1508.
198. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev*. 2004;15:457-475.
199. Fingleton B. MMPs as therapeutic targets—still a viable option? *Semin Cell Dev Biol*. 2008;19:61-68.
200. Palermo C, Joyce JA. Cysteine cathepsin proteases as pharmacological targets in cancer. *Trends Pharmacol Sci*. 2008;29:22-28.
201. Bell-McGuinn KM, Garfall AL, Bogyo M, Hanahan D, Joyce JA. Inhibition of cysteine cathepsin protease activity enhances chemotherapy regimens by decreasing tumor growth and invasiveness in a mouse model of multistage cancer. *Cancer Res*. 2007;67:7378-7385.
202. Qiang YW, Yao L, Tosato G, Rudikoff S. Insulin-like growth factor I induces migration and invasion of human multiple myeloma cells. *Blood*. 2004;103:301-308.
203. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer*. 2007;7:585-598.
204. Thompson CB, Shepard HM, O'Connor PM, et al. Enzymatic depletion of tumor hyaluronan induces antitumor responses in preclinical animal models. *Mol Cancer Ther*. 2010;9:3052-3064.

This page intentionally left blank

References

1. Friend SH, Dryja TP, Weinberg RA. Oncogenes and tumor-suppressing genes. *N Engl J Med*. 1988;318:618-622.
2. Check Hayden E. Human genome at ten: life is complicated. *Nature*. 2010;464:664-667.
3. Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature*. 2012;486:405-409.
4. Koboldt DC, Fulton RS, McLellan MD, et al. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
5. REFERENCE DELETED IN PROOFS.
6. REFERENCE DELETED IN PROOFS.
7. Sakr WA, Haas GP, Cassin BF, Pontes JE, Crissman JD. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. *J Urol*. 1993;150:379-385.
8. Nielsen M, Thomsen JL, Primdahl S, Dyreborg U, Andersen JA. Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies. *Br J Cancer*. 1987;56:814-819.
9. Weigelt B, Glas AM, Wessels LF, Witteveen AT, Peterse JL, van't Veer LJ. Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci U S A*. 2003;100:15901-15905.
10. Schmidt-Kittler O, Ragg T, Daskalakis A, et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proc Natl Acad Sci U S A*. 2003;100:7737-7742.
11. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. 2008;13:58-68.
12. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science*. 2008;321:1841-1844.
13. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med*. 2011;17:320-329.
14. Emerman JT, Pitelka DR. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro*. 1977;13:316-328.
15. Folkman J, Moscona A. Role of cell shape in growth control. *Nature*. 1978;273:345-349.
16. Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci U S A*. 1992;89:9064-9068.
17. Barcellos-Hoff MH, Aggeler J, Ram TG, Bissell MJ. Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development*. 1989;105:223-235.
18. Paget S. The distribution of secondary growths in cancer of the breast. *Cancer Metastasis Rev*. 1889;1989(8):98-101.
19. Folkman J, Kalluri R. Cancer without disease. *Nature*. 2004;427:787.
20. Rous P. An experimental comparison of transplanted tumor and a transplanted normal tissue capable of growth. *J Exp Med*. 1910;12:344-366.
21. Virchow R. *Die krankhaften Geschwülste. Dreissig Vorlesungen gehälten während des Wintersemesters 1862-1863 an der Universität zu Berlin*. Berlin: August Hirschwald. 1863.
22. Jones FS, Rous P. On the cause of the localization of secondary tumors at points of injury. *J Exp Med*. 1914;20:404-412.
23. Berenblum I, Shubik P. An experimental study of the initiating state of carcinogenesis, and a re-examination of the somatic cell mutation theory of cancer. *Br J Cancer*. 1949;3:109-118.
24. Berenblum I. The cocarcinogenic action of croton resin. *Cancer Res*. 1941;1:44-48.
25. Berenblum IA. speculative review; the probable nature of promoting action and its significance in the understanding of the mechanism of carcinogenesis. *Cancer Res*. 1954;14:471-477.
26. Rous P, Friedewald WF. The effect of chemical carcinogens on virus-induced rabbit papillomas. *J Exp Med*. 1944;79:511-538.
27. Deelman HT. The part played by injury and repair in the development of cancer; with some remarks on the growth of experimental cancers. *Proc R Soc Med*. 1927;20:1157-1158.
28. Rous P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J Exp Med*. 1911;13:397-1411.
29. Dolberg DS, Hollingsworth R, Hertle M, Bissell MJ. Wounding and its role in RSV-mediated tumor formation. *Science*. 1985;230:676-678.
30. Sieweke MH, Thompson NL, Sporn MB, Bissell MJ. Mediation of wound-related Rous sarcoma virus tumorigenesis by TGF-beta. *Science*. 1990;248:1656-1660.
31. Gimbrone Jr MA, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. *J Exp Med*. 1972;136:261-276.
32. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
33. Askanazy M. Die *Teratome nach ihrem Bau, ihrem Verlauf, ihrer Genese und im Vergleich zum experimentellen Teratoid*. *Verh Dt Pathol Ges*. 1908:39-82.
34. Mintz B, Illmensee K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc Natl Acad Sci U S A*. 1975;72:3585-3589.
35. Illmensee K, Mintz B. Totipotency and normal differentiation of single teratocarcinoma cells cloned by injection into blastocysts. *Proc Natl Acad Sci U S A*. 1976;73:549-553.
36. Brinster RL. Participation of teratocarcinoma cells in mouse embryo development. *Cancer Res*. 1976;36:3412-3414.
37. Pierce GB, Lewis SH, Miller GJ, Moritz E, Miller P. Tumorigenicity of embryonal carcinoma as an assay to study control of malignancy by the murine blastocyst. *Proc Natl Acad Sci U S A*. 1979;76:6649-6651.
38. Dolberg DS, Bissell MJ. Inability of Rous sarcoma virus to cause sarcomas in the avian embryo. *Nature*. 1984;309:552-556.
39. Stoker AW, Hatier C, Bissell MJ. The embryonic environment strongly attenuates v-src oncogenesis in mesenchymal and epithelial tissues, but not in endothelia. *J Cell Biol*. 1990;111:217-228.
40. Lee LM, Seftor EA, Bonde G, Cornell RA, Hendrix MJ. The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation. *Dev Dyn*. 2005;233:1560-1570.
41. Weaver VM, Petersen OW, Wang F, et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol*. 1997;137:231-245.
42. Sandal T, Valyi-Nagy K, Spencer VA, Folberg R, Bissell MJ, Maniotis AJ. Epigenetic reversion of breast carcinoma phenotype is accompanied by changes in DNA sequestration as measured by AluI restriction enzyme. *Am J Pathol*. 2007;170:1739-1749.
43. Muschler J, Levy D, Boudreau R, Henry M, Campbell K, Bissell MJ. A role for dystroglycan in epithelial polarization: loss of function in breast tumor cells. *Cancer Res*. 2002;62:7102-7109.
44. Wang F, Weaver VM, Petersen OW, et al. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc Natl Acad Sci U S A*. 1998;95:14821-14826.
45. Beliveau A, Mott JD, Lo A, et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. *Genes Dev*. 2010;24:2800-2811.
46. Bissell MJ, Kenny PA, Radisky DC. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. *Cold Spring Harb Symp Quant Biol*. 2005;70:343-356.
47. Onodera Y, Nam J-M, Bissell MJ. Increased sugar uptake activates oncogenic signaling via EPAC-Rap1 and O-GlcNAc pathways. *The Journal of Clinical Investigation*, In Press.
48. Bissell MJ, Radisky D. Putting tumours in context. *Nat Rev Cancer*. 2001;1:46-54.
49. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med*. 1986;315:1650-1659.
50. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860-867.
51. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol*. 2002;3:349-363.

52. Li B, Wang JH. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability*. 2011;20:108-120.
53. Jain RK. Molecular regulation of vessel maturation. *Nat Med*. 2003;9:685-693.
54. Sieweke MH, Bissell MJ. The tumor-promoting effect of wounding: a possible role for TGF-beta-induced stromal alterations. *Crit Rev Oncog*. 1994;5:297-311.
55. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
56. Sternlicht MD, Lochter A, Sympson CJ, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell*. 1999;98:137-146.
57. Ha HY, Moon HB, Nam MS, et al. Overexpression of membrane-type matrix metalloproteinase-1 gene induces mammary gland abnormalities and adenocarcinoma in transgenic mice. *Cancer Res*. 2001;61:984-990.
58. Mori H, Lo AT, Inman JL, et al. Transmembrane/cytoplasmic, rather than catalytic, domains of Mmp14 signal to MAPK activation and mammary branching morphogenesis via binding to integrin beta1. *Development*. 2013;140:343-352.
59. Sympson CJ, Talhouk RS, Alexander CM, et al. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol*. 1994;125:681-693.
60. Thomasset N, Lochter A, Sympson CJ, et al. Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development. *Am J Pathol*. 1998;153:457-467.
61. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*. 2005;436:123-127.
62. Nagy A. Cre recombinase: the universal reagent for genome tailoring. *Genesis*. 2000;26:99-109.
63. Bhowmick NA, Chytil A, Plieth D, et al. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*. 2004;303:848-851.
64. Kuperwasser C, Chavarria T, Wu M, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci U S A*. 2004;101:4966-4971.
65. Zong Y, Huang J, Sankarasharma D, et al. Stromal epigenetic dysregulation is sufficient to initiate mouse prostate cancer via paracrine Wnt signaling. *Proc Natl Acad Sci U S A*. 2012;109:E3395-E3404.
66. Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res*. 2000;60:1254-1260.
67. Monnier Y, Farmer P, Bieler G, et al. CYR61 and alphaVbeta5 integrin cooperate to promote invasion and metastasis of tumors growing in preirradiated stroma. *Cancer Res*. 2008;68:7323-7331.
68. Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol*. 2011;3:2-27.
69. Ghajar CM, Bissell MJ. Extracellular matrix control of mammary gland morphogenesis and tumorigenesis: insights from imaging. *Histochem Cell Biol*. 2008;130:1105-1118.
70. Bissell MJ, Hall HG, Parry G. How does the extracellular matrix direct gene expression? *J Theor Biol*. 1982;99:31-68.
71. Boudreau N, Sympson CJ, Werb Z, Bissell MJ. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science*. 1995;267:891-893.
72. Gudjonsson T, Ronnov-Jessen L, Villadsen R, Rank F, Bissell MJ, Petersen OW. Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *J Cell Sci*. 2002;115:39-50.
73. Xu R, Nelson CM, Muschler JL, Veiseh M, Vonderhaar BK, Bissell MJ. Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. *J Cell Biol*. 2009;184:57-66.
74. Streuli CH, Schmidhauser C, Bailey N, et al. Laminin mediates tissue-specific gene expression in mammary epithelia. *J Cell Biol*. 1995;129:591-603.
75. Roskelley CD, Desprez PY, Bissell MJ. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc Natl Acad Sci U S A*. 1994;91:12378-12382.
76. Streuli CH, Bailey N, Bissell MJ. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J Cell Biol*. 1991;115:1383-1395.
77. Hu M, Yao J, Carroll DK, et al. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*. 2008;13:394-406.
78. Sappino AP, Skalli O, Jackson B, Schurch W, Gabbiani G. Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int J Cancer*. 1988;41:707-712.
79. Ronnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest*. 1995;95:859-873.
80. Spaeth EL, Dembinski JL, Sasser AK, et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One*. 2009;4: e4992.
81. Petersen OW, Nielsen HL, Gudjonsson T, et al. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol*. 2003;162:391-402.
82. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res*. 2007;67:10123-10128.
83. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6:392-401.
84. Ronnov-Jessen L, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest*. 1993;68:696-707.
85. Gao MQ, Kim BG, Kang S, et al. Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial-mesenchymal transition-like state in breast cancer cells in vitro. *J Cell Sci*. 2010;123:3507-3514.
86. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res*. 1999;59:5002-5011.
87. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.
88. Schauer IG, Sood AK, Mok S, Liu J. Cancer-associated fibroblasts and their putative role in potentiating the initiation and development of epithelial ovarian cancer. *Neoplasia*. 2011;13:393-405.
89. Bachem MG, Schunemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128:907-921.
90. Mazzocca A, Fransvea E, Dituri F, Lupo L, Antonaci S, Giannelli G. Down-regulation of connective tissue growth factor by inhibition of transforming growth factor beta blocks the tumor-stroma cross-talk and tumor progression in hepatocellular carcinoma. *Hepatology*. 2010;51:523-534.
91. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell*. 2010;17:135-147.
92. Kojima Y, Acar A, Eaton EN, et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A*. 2010;107:20009-20014.
93. Chang HY, Chi JT, Dudoit S, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A*. 2002;99:12877-12882.
94. Qiu W, Hu M, Sridhar A, et al. No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas. *Nat Genet*. 2008;40:650-655.
95. Trimboli AJ, Cantemir-Stone CZ, Li F, et al. Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature*. 2009;461:1084-1091.
96. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res*. 2010;70:9621-9630.
97. Gaggioli C, Hooper S, Hidalgo-Carcedo C, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol*. 2007;9:1392-1400.
98. Fisher KE, Sacharidou A, Stratman AN, et al. MT1-MMP- and Cdc42-dependent signaling co-regulate cell invasion and tunnel formation in 3D collagen matrices. *J Cell Sci*. 2009;122:4558-4569.

99. Wolf K, Mazo I, Leung H, et al. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol*. 2003;160:267-277.
100. Correia AL, Mori H, Chen EI, Schmitt FC, Bissell MJ. The hemopexin domain of MMP3 is responsible for mammary epithelial invasion and morphogenesis through extracellular interaction with HSP90beta. *Genes Dev*. 2013;27:805-817.
101. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer*. 2006;6:227-239.
102. Jain RK. Transport of molecules in the tumor interstitium: a review. *Cancer Res*. 1987;47:3039-3051.
103. Paszek MJ, Zahir N, Johnson KR, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell*. 2005;8:241-254.
104. Alcaraz J, Xu R, Mori H, et al. Laminin and biomimetic extracellular elasticity enhance functional differentiation in mammary epithelia. *EMBO J*. 2008;27:2829-2838.
105. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009;139:891-906.
106. Wozniak MA, Desai R, Solski PA, Der CJ, Keely PJ. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol*. 2003;163:583-595.
107. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med*. 2006;4:38.
108. Conklin MW, Eickhoff JC, Riching KM, et al. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol*. 2011;178:1221-1232.
109. Maniotis AJ, Folberg R, Hess A, et al. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol*. 1999;155:739-752.
110. Folberg R, Pe'er J, Gruman LM, et al. The morphologic characteristics of tumor blood vessels as a marker of tumor progression in primary human uveal melanoma: a matched case-control study. *Hum Pathol*. 1992;23:1298-1305.
111. Provenzano PP, Inman DR, Eliceiri KW, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med*. 2008;6:11.
112. Jain RK. Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev*. 2001;46:149-168.
113. Ng CP, Hinz B, Swartz MA. Interstitial fluid flow induces myofibroblast differentiation and collagen alignment in vitro. *J Cell Sci*. 2005;118:4731-4739.
114. Wipff PJ, Rifkin DB, Meister JJ, Hinz B. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol*. 2007;179:1311-1323.
115. Goetz JG, Joshi B, Lajoie P, et al. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. *J Cell Biol*. 2008;180:1261-1275.
116. Goetz JG, Minguet S, Navarro-Lerida I, et al. Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell*. 2011;146:148-163.
117. Sloan EK, Ciocca DR, Pouliot N, et al. Stromal cell expression of caveolin-1 predicts outcome in breast cancer. *Am J Pathol*. 2009;174:2035-2043.
118. Witkiewicz AK, Dasgupta A, Sotgia F, et al. An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. *Am J Pathol*. 2009;174:2023-2034.
119. Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol*. 2009;9:259-270.
120. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 2004;4:71-78.
121. Grey A, Chen Y, Paliwal I, Carlberg K, Insogna K. Evidence for a functional association between phosphatidylinositol 3-kinase and c-src in the spreading response of osteoclasts to colony-stimulating factor-1. *Endocrinology*. 2000;141:2129-2138.
122. O'Brien J, Martinson H, Durand-Rougely C, Schedin P. Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development*. 2012;139:269-275.
123. Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn*. 2006;235:3222-3229.
124. Michaelson MD, Bieri PL, Mehler MF, et al. CSF-1 deficiency in mice results in abnormal brain development. *Development*. 1996;122:2661-2672.
125. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141:39-51.
126. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med*. 2001;193:727-740.
127. DeNardo DG, Barreto JB, Andreu P, et al. CD4$^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*. 2009;16:91-102.
128. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
129. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res*. 2007;67:5064-5066.
130. Lin EY, Li JF, Gnatovskiy L, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*. 2006;66:11238-11246.
131. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 2004;64:7022-7029.
132. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science*. 2013;339:286-291.
133. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2002;2:563-572.
134. Lammert E, Cleaver O, Melton D. Induction of pancreatic differentiation by signals from blood vessels. *Science*. 2001;294:564-567.
135. Matsumoto K, Yoshitomi H, Rossant J, Zaret KS. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science*. 2001;294:559-563.
136. Mathieu C, Sii-Felice K, Fouchet P, et al. Endothelial cell-derived bone morphogenetic proteins control proliferation of neural stem/progenitor cells. *Mol Cell Neurosci*. 2008;38:569-577.
137. Shen Q, Goderie SK, Jin L, et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science*. 2004;304:1338-1340.
138. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer*. 10:138-146.
139. Butler JM, Nolan DJ, Vertes EL, et al. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*. 2010;6:251-264.
140. Kobayashi H, Butler JM, O'Donnell R, et al. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol*. 12:1046-1056.
141. Kienast Y, von Baumgarten L, Fuhrmann M, et al. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med*. 16:116-122.
142. Lathia JD, Gallagher J, Heddleston JM, et al. Integrin alpha 6 regulates glioblastoma stem cells. *Cell Stem Cell*. 2010;6:421-432.
143. Zhu TS, Costello MA, Talsma CE, et al. Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer Res*. 2011;71:6061-6072.
144. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. 2007;11:69-82.
145. Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. *Genes Dev*. 2011;25:2559-2572.
146. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. 2001;7:1194-1201.
147. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
148. Campbell JP, Karolak MR, Ma Y, et al. Stimulation of host bone marrow stromal cells by sympathetic nerves promotes breast cancer bone metastasis in mice. *PLoS Biol*. 2012;10: e1001363.
149. Chandler EM, Seo BR, Califano JP, et al. Implanted adipose progenitor cells as physicochemical regulators of breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:9786-9791.

150. Lyden D, Welch DR, Psaila B, eds. *Cancer Metastasis: Biologic Basis and Therapeutics*. New York, NY: Cambridge University Press; 2011.
151. Suzuki M, Mose ES, Montel V, Tarin D. Dormant cancer cells retrieved from metastasis-free organs regain tumorigenic and metastatic potency. *Am J Pathol*. 2006;169:673-681.
152. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7:834-846.
153. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-827.
154. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001;410:50-56.
155. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol*. 2000;2:737-744.
156. Erler JT, Bennewith KL, Cox TR, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell*. 2009;15:35-44.
157. Hiratsuka S, Watanabe A, Sakurai Y, et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol*. 2008;10:1349-1355.
158. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood*. 2007;109:1010-1017.
159. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med*. 2005;201:1089-1099.
160. Peinado H, Lavotshkin S, Lyden D. The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol*. 2011;21:139-146.
161. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18:883-891.
162. Jones ML, Siddiqui J, Pienta KJ, Getzenberg RH. Circulating fibroblast-like cells in men with metastatic prostate cancer. *Prostate*. 2013;73:176-181.
163. Duda DG, Duyverman AM, Kohno M, et al. Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci U S A*. 2010;107:21677-21682.
164. Xu Z, Vonlaufen A, Phillips PA, et al. Role of pancreatic stellate cells in pancreatic cancer metastasis. *Am J Pathol*. 2010;177:2585-2596.
165. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. 2009;9:285-293.
166. Bos PD, Zhang XH, Nadal C, et al. Genes that mediate breast cancer metastasis to the brain. *Nature*. 2009;459:1005-1009.
167. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;436:518-524.
168. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 2003;3:537-549.
169. Oskarsson T, Acharyya S, Zhang XH, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. 2011;17:867-874.
170. Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2012;481:85-89.
171. Kim S, Takahashi H, Lin WW, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature*. 2009;457:102-106.
172. Barkan D, El Touny LH, Michalowski AM, et al. Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment. *Cancer Res*. 2010;70:5706-5716.
173. Studebaker AW, Storci G, Werbeck JL, et al. Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. *Cancer Res*. 2008;68:9087-9095.
174. Gil-Bernabe AM, Ferjancic S, Tlalka M, et al. Recruitment of monocytes/macrophages by tissue factor-mediated coagulation is essential for metastatic cell survival and premetastatic niche establishment in mice. *Blood*. 2012;119:3164-3175.
175. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med*. 2004;10:789-799.
176. Jain RK. Understanding barriers to drug delivery: high resolution in vivo imaging is key. *Clin Cancer Res*. 1999;5:1605-1606.
177. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. 2005;15:102-111.
178. Netti PA, Berk DA, Swartz MA, Grodzinsky AJ, Jain RK. Role of extracellular matrix assembly in interstitial transport in solid tumors. *Cancer Res*. 2000;60:2497-2503.
179. Provenzano PP, Cuevas C, Chang AE, Goel VK, Von Hoff DD, Hingorani SR. Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma. *Cancer Cell*. 2012;21:418-429.
180. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A*. 2011;108:2909-2914.
181. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*. 1994;124:619-626.
182. Fridman R, Giaccone G, Kanemoto T, Martin GR, Gazdar AF, Mulshine JL. Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. *Proc Natl Acad Sci U S A*. 1990;87:6698-6702.
183. Weaver VM, Lelievre S, Lakins JN, et al. beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell*. 2002;2:205-216.
184. Nam JM, Onodera Y, Bissell MJ, Park CC. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. *Cancer Res*. 2010;70:5238-5248.
185. Sherman-Baust CA, Weeraratna AT, Rangel LB, et al. Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. *Cancer Cell*. 2003;3:377-386.
186. Serebriiskii I, Castello-Cros R, Lamb A, Golemis EA, Cukierman E. Fibroblast-derived 3D matrix differentially regulates the growth and drug-responsiveness of human cancer cells. *Matrix Biol*. 2008;27:573-585.
187. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. 2008;6:2853-2868.
188. Acharyya S, Oskarsson T, Vanharanta S, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell*. 2012;150:165-178.
189. Nakasone ES, Askautrud HA, Kees T, et al. Imaging tumor-stroma interactions during chemotherapy reveals contributions of the microenvironment to resistance. *Cancer Cell*. 2012;21:488-503.
190. Shree T, Olson OC, Elie BT, et al. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev*. 2011;25:2465-2479.
191. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov*. 2004;3:391-400.
192. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*. 1997;88:277-285.
193. Kupsch P, Henning BF, Passarge K, et al. Results of a phase I trial of sorafenib (BAY 43-9006) in combination with oxaliplatin in patients with refractory solid tumors, including colorectal cancer. *Clin Colorectal Cancer*. 2005;5:188-196.
194. Pan BS, Chan GK, Chenard M, et al. MK-2461, a novel multitargeted kinase inhibitor, preferentially inhibits the activated c-Met receptor. *Cancer Res*. 2010;70:1524-1533.
195. Wolf AM, Rumpold H, Tilg H, Gastl G, Gunsilius E, Wolf D. The effect of zoledronic acid on the function and differentiation of myeloid cells. *Haematologica*. 2006;91:1165-1171.
196. Veltman JD, Lambers ME, van Nimwegen M, et al. Zoledronic acid impairs myeloid differentiation to tumour-associated macrophages in mesothelioma. *Br J Cancer*. 2010;103:629-641.
197. Teitelbaum SL. Bone resorption by osteoclasts. *Science*. 2000;289:1504-1508.
198. Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev*. 2004;15:457-475.
199. Fingleton B. MMPs as therapeutic targets—still a viable option? *Semin Cell Dev Biol*. 2008;19:61-68.
200. Palermo C, Joyce JA. Cysteine cathepsin proteases as pharmacological targets in cancer. *Trends Pharmacol Sci*. 2008;29:22-28.

201. Bell-McGuinn KM, Garfall AL, Bogyo M, Hanahan D, Joyce JA. Inhibition of cysteine cathepsin protease activity enhances chemotherapy regimens by decreasing tumor growth and invasiveness in a mouse model of multistage cancer. *Cancer Res*. 2007;67:7378-7385.
202. Qiang YW, Yao L, Tosato G, Rudikoff S. Insulin-like growth factor I induces migration and invasion of human multiple myeloma cells. *Blood*. 2004;103:301-308.
203. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer*. 2007;7:585-598.
204. Thompson CB, Shepard HM, O'Connor PM, et al. Enzymatic depletion of tumor hyaluronan induces antitumor responses in preclinical animal models. *Mol Cancer Ther*. 2010;9:3052-3064.

Brian Keith and M. Celeste Simon

17 Tumor Angiogenesis

Solid tumors require a vascular system to grow beyond about 2 mm in diameter, a size at which diffusion of oxygen and nutrients is limiting. The establishment of a tumor vasculature through the process of *angiogenesis* overcomes these limitations, while also providing a conduit through which cancer cells can metastasize. The close association between tumor growth and increased vascularity was described in the 19th century by several researchers,[1] including the pathologist Rudolf Virchow, who also first proposed a link between chronic inflammation and cancer.[2] An important experimental advance in angiogenic research came in the 1920s, when transparent chambers were first used to observe the growth of vessels into tumors in animals in real time.[1] In a seminal study published in 1950, Algire and colleagues[3] used transparent chambers to follow vessel recruitment to a variety of normal and malignant tissues transplanted into mice. Their studies provided some of the first observations that transplanted tumor tissue, in contrast to normal tissue, induced the development of an extensive vascular bed; moreover, this angiogenic response preceded rapid tumor growth. The authors succinctly stated the now-axiomatic idea that "the rapid growth of tumor explants is dependent on the development of a rich vascular supply."[3] Judah Folkman's proposal in 1971 that tumor growth might be inhibited, or even reversed, by blocking tumor angiogenesis[4] sparked a remarkable flurry of activity in basic and clinical research. A milestone in angiogenic cancer therapy was passed in 2003, when the U.S. Food and Drug Administration (FDA) approved the anti-angiogenic monoclonal antibody Avastin (bevacizumab) as a first-line treatment for metastatic colorectal cancer.

This chapter provides a general overview of tumor angiogenesis, highlighting specific molecular pathways that regulate this process, and discusses the ongoing development and clinical evaluation of anti-angiogenic therapies. It is organized into sections that describe (1) the development of vascular structures in normal and malignant tissues, (2) the signaling pathways that regulate their formation, and (3) the therapeutic strategies that underlie ongoing drug development, as well as the current results of clinical trials using anti-angiogenic therapies. Finally, we discuss some of the remaining questions and challenges that are likely to drive angiogenic research in the coming years.

Vascular Development

Normal Vascular Development

Development of the vascular system is one of the first events in embryonic organogenesis. Mesoderm-derived *vascular endothelial cells (ECs)* generate lumen-containing tubular structures that form the basic functional unit of blood vessels. Initially, vascular networks form independently in the yolk sac and the embryo, and then connect to generate a closed circulatory system. In a process known as *vasculogenesis*, endothelial cell progenitors (*angioblasts*) and their derivative ECs aggregate de novo in the yolk sac to form a primitive vascular network or plexus of approximately uniform dimensions (Figure 17-1). Subsequent *angiogenesis* occurs through vessel *sprouting*, in which ECs from existing vessels respond to angiogenic signals by degrading their basement membrane, loosening their association with support cells, altering their morphology, and proliferating. These ECs migrate in response to chemotactic signals and coalesce to form new vessels that connect to the existing vasculature. The coordinated recruitment of supporting mural cells, including *pericytes* and *smooth muscle cells*, results in vessel maturation. In a parallel mechanism termed *intussusception*, columns of endothelial cells create a barrier in the lumen of a preexisting vessel, thus partitioning it into multiple independent vessels[5,6] (Figure 17-2). This complex series of events produces a closed, highly arborized system of larger and smaller vessels including arteries, veins, and capillaries.

In contrast to the yolk sac, angioblasts in the embryo migrate along specific pathways and aggregate directly to form the dorsal aorta and posterior cardinal vein, without

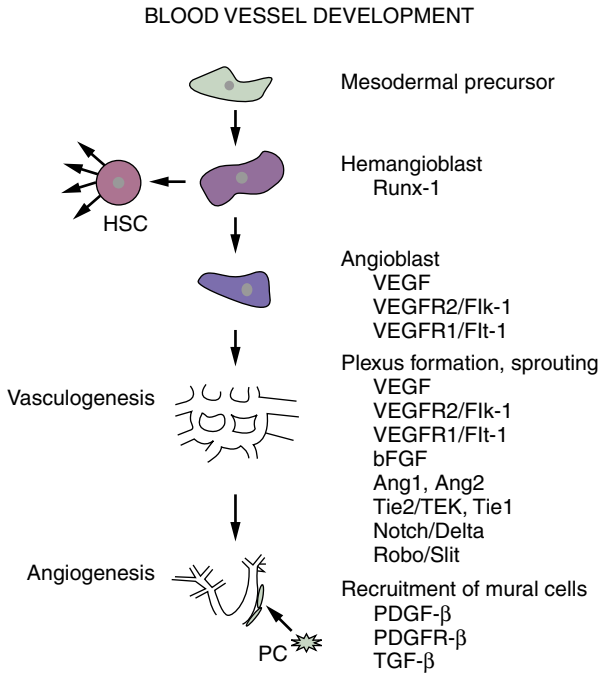


Figure 17-1 Major events in vascular development Some of the critical signaling molecules and receptors are shown corresponding to the cells or processes in which they are known to play a role. Vascular progenitors are derived from vascular endothelial growth receptor-2 (VEGFR2/Flk-1)-positive cells in the lateral plate mesoderm. Hemogenic endothelial cells give rise to hematopoietic stem cells (HSCs) and vascular endothelial precursors (angioblasts). In the yolk sac, angioblasts align to generate a primary capillary plexus (vasculogenesis). Vessels in this plexus grow primarily by sprouting, which involves endothelial cell proliferation and migration (angiogenesis), and eventually connect to vessels in the embryo to form a closed vascular system. Vasculogenesis and angiogenesis are both highly dependent on VEGF, angiopoietins, and their receptors, along with many other signaling molecules (see figure and text). Maturation of the vascular system requires remodeling of the vascular network into large and small vessels, along with the recruitment of supporting mural cells (pericytes and smooth-muscle cells). *Ang*, Angiopoietin; *Notch/Delta4*, Notch receptor/Delta4 ligand; *PC*, pericyte; *PDGF-β*, platelet-derived growth factor β; *PDGFR-β*, PDGF receptor β; *Robo/Slit*, roundabout receptor/slit ligand; *TGF-β*, transforming growth factor-β; *Tie2/Tek* and *Tie1*, Tie family of endothelial receptor tyrosine kinases. *(Adapted from Risau W. Mechanisms of angiogenesis.* Nature. *1997;386:671-674).*

passing through an intermediate plexus phase.[7] These vessels undergo subsequent remodeling and ultimately connect to the extra-embryonic yolk sac vessels to form a mature vascular system. Interestingly, vascular development is also intimately associated with the development of hematopoietic cell lineages, as *hematopoietic stem cells* (HSCs) have been shown to arise from the *hemogenic endothelium*, which comprises Runx-1 expressing ECs restricted to the ventral portion of the developing dorsal aorta (see Figure 17-1).[8]

The vessels of the parallel *lymphatic* system collect and return interstitial fluids, particulates, and extravasated cells to the venous circulation. Lymphatic vessels differ from blood vessels in that lymphatic capillaries have internal membranous valves that prevent fluid backflow, and they are generally not surrounded by support cells.[9] Lymphatic ECs are derived from primitive veins and express and respond to a different spectrum of receptors and signaling molecules than ECs in blood vessels (Ref. 9, and see later discussion). The ability of cancer cells to invade lymphatics and collect in lymph nodes, complex organs involved in local immune surveillance, is an important indicator of tumor metastasis. It is likely that the lymphatic vessels at the periphery of a solid tumor are most directly involved in metastasis, as interstitial pressure within the tumor often leads to vessel collapse.[5,9] Recent evidence supports the idea that lymphatic ECs may secrete chemokines that attract tumor cells and may therefore participate more actively in metastasis than was previously recognized.[10]

In adult humans and mice, there is little regular angiogenic activity, with the notable exception of the female reproductive system. Localized angiogenesis is, however, an important aspect of normal wound healing, and *inflammatory cells* including macrophages, neutrophils, and mast cells, as well as activated resident fibroblasts, are an important source of angiogenic modulators during wound repair. Recently, it was shown that macrophages directly bind angiogenic ECs and promote the formation of anastomoses between them.[11] On remodeling and fusion with the surrounding vasculature, these new vessels restore normal blood supply to the wounded area. These infiltrating stromal cells also represent an important component of many solid tumors, where they can produce angiogenic factors as part of what may be considered an aberrant wound healing response, leading to the idea that tumors represent "wounds that never heal."[12] Genetic experiments using multiple murine cancer models have established that tumor-associated macrophages play a critical role in driving tumor angiogenesis and metastasis.[12]

Rapid growth of any tissue (embryos, neoplasias, adipose tissue, regenerating liver, etc.) invariably requires a supply of oxygen, nutrients, and hormones and is typically accompanied by active angiogenesis. Consequently, angiogenesis can be seen as a genetically programmed, dynamic process that can be activated locally in response to stimulatory signals. The fact that most blood vessels in the adult body are quiescent has been proposed as an advantage of anti-angiogenic strategies, which typically target actively dividing ECs, as these drugs may be less generally toxic to quiescent ECs lining established vessels.

Tumor Vasculature

The blood vessels found in solid tumors are notable for being highly disorganized compared to those of normal organs and are characterized by tortuous and misshapen vessels that sometimes terminate in open-ended blood lakes[5,13] (Figure 17-3). These aberrant vessels are thought to result from dysregulated angiogenic signaling in the tumor bed, as a result

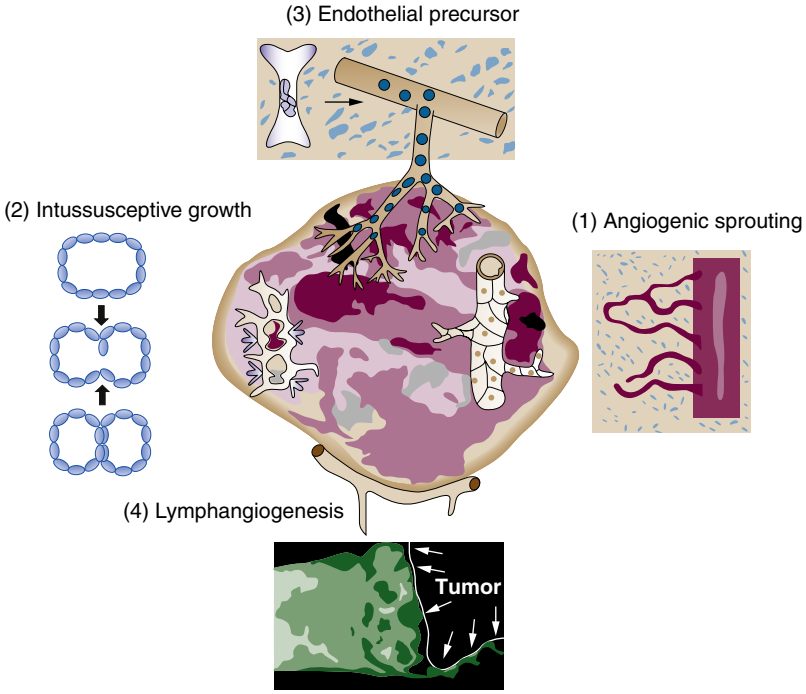


Figure 17-2 Cellular mechanisms of tumor angiogenesis Tumor vessels grow by multiple mechanisms, some of which are formally similar to those observed in normal vascular development: (1) budding of endothelial sprouts and formation of bridges (angiogenesis) and (2) insertion of interstitial tissue columns into the lumen of preexisting vessels (intussusception). In contrast to normal vascular development, the signaling events controlling these events are often highly disordered, resulting in chaotic vascular organization, uneven blood flow, and localized hypoxia. In addition, endothelial cell precursors home to tumors from the bone marrow or peripheral blood (3) where they can contribute, either directly or indirectly, to the endothelial lining of tumor vessels. Lymphatic vessels (4) around tumors drain interstitial fluid and also provide a gateway for metastasizing tumor cells. *(Reproduced from Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases.* Nature. *2000;407:249-257).*

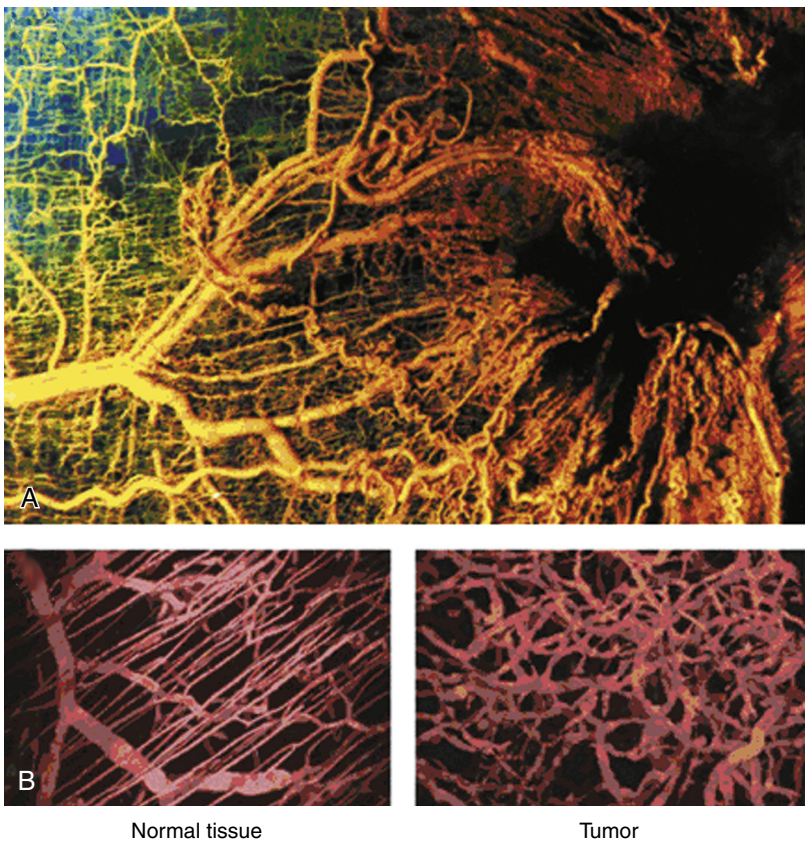


Figure 17-3 The highly disorganized nature of tumor vasculature can be visualized by generating a polymer cast before fixation **(A)** or using intravital imaging techniques that reveal functional vessels in live tissues **(B)**. As opposed to the clearly ordered arrangement of vessels in normal tissue, the chaotic nature of tumor vessels reflects the disrupted balance of pro- and anti-angiogenic factors generated by tumor and stromal cells. *(Reproduced from Weinberg RA.* The Biology of Cancer. *New York, NY: Garland Science; 2007:562).*

of oncogene activation and tumor suppressor loss. Microscopic analysis of tumor vessels reveals disrupted junctions between tumor ECs and reduced or inconsistent coverage by pericytes, which helps explain the increased permeability characteristic of tumor vessels.[14] The origin of some tumor ECs is also controversial: In addition to ECs recruited through sprouting of preexisting vessels, growing evidence supports a role of circulating endothelial progenitor cells (EPCs) that either differentiate into endothelial-like cells or promote expansion of bona fide ECs (see Figure 17-2). The precise cellular origins and complexity of these cells remain controversial, and the degree to which murine EPCs actually contribute directly to the lining of new tumor vessels varies considerably, depending on the model used, genetic background, and other factors.[15,16] In addition, bone marrow–derived myeloid cells contribute to tumor angiogenesis; these cells have been reported to express a variety of cell surface markers, including those common to endothelial cells (Tie-2) and myeloid cells (CD11b, Gr-1), and may function by providing paracrine angiogenic signals.[17,18] It is interesting to note that genetic ablation of bone marrow–derived Tie-2 expressing monocytes (TEMs), in particular, has profound effects on tumor angiogenesis in mice (see Refs. 19 and 20, and references therein).

Tumors often display sluggish, uneven, and highly variable patterns of blood flow,[13] as well as direct arteriole-venule shunts.[21] Tumor vessels also differ from normal vasculature in being exposed to an acidic microenvironment characterized by oxygen and nutrient deprivation. In rapidly growing tumors, aberrant angiogenic regulation and high interstitial pressure can produce regions of localized anoxia and/or ischemia. This typically results in pockets of necrosis surrounded by a penumbra of hypoxic but living cells. Severely hypoxic conditions are thought to protect tumor cells from radiation therapy, which depends on the generation of reactive oxygen intermediates to kill tumor cells. Moreover, hypoxic regions in tumors appear to select for highly malignant cancer cells.[22] In particular, hypoxia directly promotes angiogenic signaling in tumors, as discussed in more detail later.[23]

The degree to which tumors generate vascular beds is often expressed as *microvessel*, or *mean vessel*, *density (MVD)*, which can vary widely within a given tumor and between tumors of similar or different tissues. MVD is traditionally determined by staining tumor sections with antibodies raised against proteins expressed on ECs, including CD31 (PECAM), CD34, and von Willebrand factor. Clinical studies have demonstrated that MVD is a useful prognostic indicator for a wide array of cancers, including breast, prostate, non–small-cell lung, gastrointestinal, and even hematological tumors.[24] It is important to note, however, that not all tumor vessels are functional and that MVD may greatly exceed the basic metabolic requirements of a growing tumor. The striking functional heterogeneity of vessels within a tumor, and the ability of many cancer cells to withstand severe hypoxia, glucose deprivation, and tissue acidity, makes it difficult to assess the effects of angiogenesis-based therapies based solely on MVD.[24]

Critical Signaling Factors—Targets for Therapy

Over the past 15 years, work from many laboratories has demonstrated that vascular development in normal tissues is under elaborate genetic and molecular control. Many of the signaling molecules that regulate normal developmental angiogenesis have also been shown to drive angiogenesis in cancer and other pathophysiological conditions, although their expression and function in tumors are often highly uncoordinated. A growing list of molecules has been shown to regulate different aspects of developmental and pathological angiogenesis. Primary among these is the family of vascular endothelial growth factors (*VEGF*s) that, along with their receptors, regulate endothelial cell proliferation, survival, and function. The vascular-specific *angiopoietins* and their receptor tyrosine kinases also play important roles in angiogenic remodeling. In addition, vascular development is regulated by signaling pathways familiar from other developmental processes, including fibroblast growth factors (in particular, basic or *bFGF*), transforming growth factor beta *(TGF-β)*, *Notch* and its ligand Delta-like ligand 4 *(Dll4)*, and platelet-derived growth factor *(PDGF)*. In addition, a number of molecules originally implicated in controlling axon guidance, including the *semaphorins*, *netrins*, and *Robo/slit*, have been shown to contribute to vascular development.[7,25] Finally, the Notch pathway, along with the *EphB4/ephrinB2* signaling system, has been shown to control specification of arteries and veins (see Refs. 7 and 25, and references therein). Our understanding of the mechanisms by which these genes and pathways regulate angiogenesis is based largely on genetic "knockout" experiments in mice, often confirmed by in vitro cell-based assays or in experimental tumors. How this complex array of signaling pathways is coordinated to regulate angiogenic events in normal organogenesis and disease is a focus of intensive research. The discovery of endogenous angiogenic inhibitors, including *thrombospondin-1*, *endostatin*, *tumstatin*, and others, provided strong support for the idea that angiogenesis regulated by the balance between pro- and anti-angiogenic factors.[26] In this section, we discuss the molecular biology and function of a small subset of pro-angiogenic and anti-angiogenic factors that show particular promise as targets for cancer therapies.

Pro-angiogenic Factors

VEGF

Vascular endothelial growth factor (also known as *VEGF-A*) is among the most potent angiogenic factors described and stimulates EC proliferation, survival, chemotaxis, and vessel permeability. VEGF belongs to a family of structurally related growth factors that includes placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. VEGF is a homodimeric glycoprotein of 45 kDa and is expressed in four different molecular-weight forms—VEGF-121, VEGF-165, VEGF-189, and VEGF-206—produced by differential mRNA splicing. VEGF-121 is diffusible, whereas the other forms bind to heparin and heparin proteoglycans in the extracellular matrix (ECM) and on cell surfaces. These bound forms are released through the action of proteases, including plasmin and matrix metalloproteases (MMPs), which are produced by tumor cells and/or by activated stromal cells. Interestingly, VEGF was first identified as vascular permeability factor (VPF) based on its ability to increase the leakage of fluid and plasma proteins from blood vessels (see Refs. 1, 5, 14, and 27 for details). These leaked proteins provide an ECM for migrating ECs, and their release into interstitial spaces represents an early step in angiogenesis. The central importance of VEGF in regulating angiogenesis became clear through genetic targeting experiments in mice. Loss of only one *Vegf* allele resulted in lethality at embryonic day 9.5 (E9.5), characterized by a reduction in ECs and abnormal vessel morphology.[28,29] Embryos lacking both *Vegf* alleles died even earlier (E8.5) and displayed a complete absence of the dorsal aorta and other vascular structures.

VEGF mediates its effects by binding its cognate receptor tyrosine kinases, VEGFR1 (also called Flt-1) and VEGFR-2 (also called Flk-1 or KDR). Binding of VEGF to VEGFR-2/Flk-1 triggers receptor autophosphorylation and robustly activates several downstream signaling pathways (including phosphoinositide 3-kinase [PI3K], Src, and protein kinase C [PKC]), leading to rapid and profound effects on EC proliferation, survival, migration and gene expression.[7,30] Genetic ablation of Flk-1 in mice caused embryonic lethality at E8.5 that correlated with a loss of normal vascular structures and hematopoietic cells, consistent with the bipotential fate of *hemogenic endothelial* cells.[8,31] Subsequent studies have confirmed the importance of VEGF and VEGFR-2/Flk-1 in hematopoietic development (Ref. 7 and references therein). Although VEGFR-1/Flt-1 also binds VEGF, its major angiogenic function may be to modulate the amount of VEGF available to bind to VEGFR-2/Flk-1.[14] Deletion of the gene encoding murine VEGFR-1/Flt-1 resulted in embryonic lethality; however, this lethality was rescued by transgenic expression of a truncated VEGFR-1/Flt-1 protein that lacked its cytoplasmic signaling domain. Although these results argue strongly that VEGFR-1/Flt-1 acts as a nonsignaling sink for free VEGF, subsequent studies indicate that it can, in fact, modulate pathophysiological angiogenesis, possibly by intermolecular phosphorylation of VEGFR-2/Flk-1.[9] Neuropilins 1 and 2 can also act as a sink for VEGF and appear to function, at least in part, by presenting VEGF to VEGFR-2/Flk-1 or by modulating its effective free concentration.[32]

The central role of VEGF signaling in tumor angiogenesis has been clearly demonstrated in a wide variety of experimental models, including VEGF overexpression in tumor or host cells, treatment with recombinant VEGF, increased VEGF expression in response to oncogene activation, or inhibition by antisense VEGF oligonucleotides or anti-VEGF antibodies.[1,25] Furthermore, many oncoproteins (including KRAS, HER2, FOS, and TRKB), tumor suppressor proteins (including pVHL and p53), and growth factors (including PDGF, bFGF, and TGF-β) regulate angiogenesis, partly by inducing the expression of VEGF either directly or indirectly.[25]

The *von Hippel–Lindau* (pVHL) tumor suppressor is a particularly interesting case in point. Patients with VHL disease, a hereditary cancer syndrome, develop a variety of tumor types including highly vascularized renal clear cell carcinomas, cerebral hemangioblastomas, and retinal hemangiomas. The pVHL protein functions as an E3 ubiquitin ligase that targets the *hypoxia inducible factor (HIF)* subunits HIF-1α and HIF-2α for oxygen-dependent degradation via the 26S proteasome.[33] HIF-1α and HIF-2α play a predominant role in hypoxic responses,[34] and their activity is controlled in a similar oxygen-dependent fashion. Both proteins regulate the expression of target genes that mediate adaptive responses to hypoxic stress, including those encoding VEGF and many other angiogenic factors[23,35] (see Figures 17-3 and 17-4). When pVHL expression or function is lost, cells can no longer degrade the HIF-α subunits under conditions of abundant oxygen, leading to constitutive expression of VEGF and other HIF target genes, thereby promoting tumor angiogenesis (Figure 17-5). Both HIF-α subunits are often overexpressed in cancer cells as a consequence of oncogene activation, tumor suppressor loss, or tumor hypoxia. The close spatial overlap between HIF-α protein accumulation and VEGF expression in hypoxic tumor cells is a further indication that HIF-dependent VEGF expression is an important aspect of tumor angiogenesis (see Figure 17-3).

Both HIF-1α and HIF-2α bind to the *Vegf* gene promoter and can activate VEGF expression independently; hence, deletion of either subunit has relatively subtle effects on embryonic VEGF expression, despite the fact that both mutations are embryonically lethal.[23,36-38] Targeted deletion of the common binding partner (HIF-1β or ARNT), however, resulted in early embryonic lethality with substantial

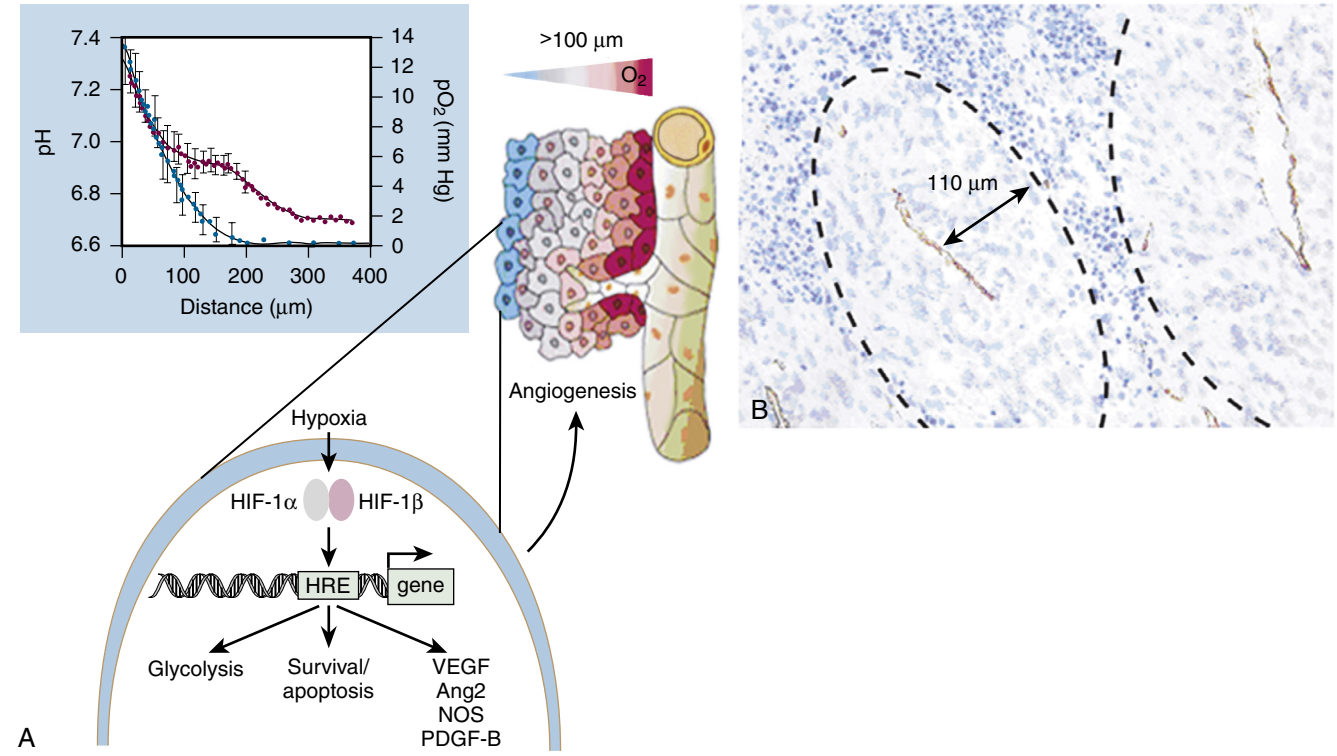


Figure 17-4 **(A)** Because of the irregular pattern and organization of the tumor vasculature, some cells in tumors are located more than 100 m (the diffusion limit for oxygen) away from blood vessels and become hypoxic (red-to-blue gradient indicates progressive hypoxia). Tumor cells survive fluctuations in oxygen tensions, in part because clones are selected in hypoxic tumors that switch to a pro-angiogenic phenotype. HIFs increase transcription of several angiogenic genes (for example, genes encoding VEGF, PDGF-BB, and nitric oxide synthase [NOS]). HIFs also affect cellular survival/apoptosis pathways. *Inset:* Relationship between the distance of tumor cells from nearby vessels and their degree of hypoxia (*blue* symbols) and acidosis (*red* symbols). *(Reproduced from Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases.* Nature. *2000;407:249-257).* **(B)** Section of rat prostatic carcinoma in which vessels were identified by CD31 immunostaining. A "cuff" of viable cells surrounds each capillary, beyond which regions of necrosis are evident. *(Reproduced from Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us.* J Natl Cancer Inst. *2002;94:883-893).*

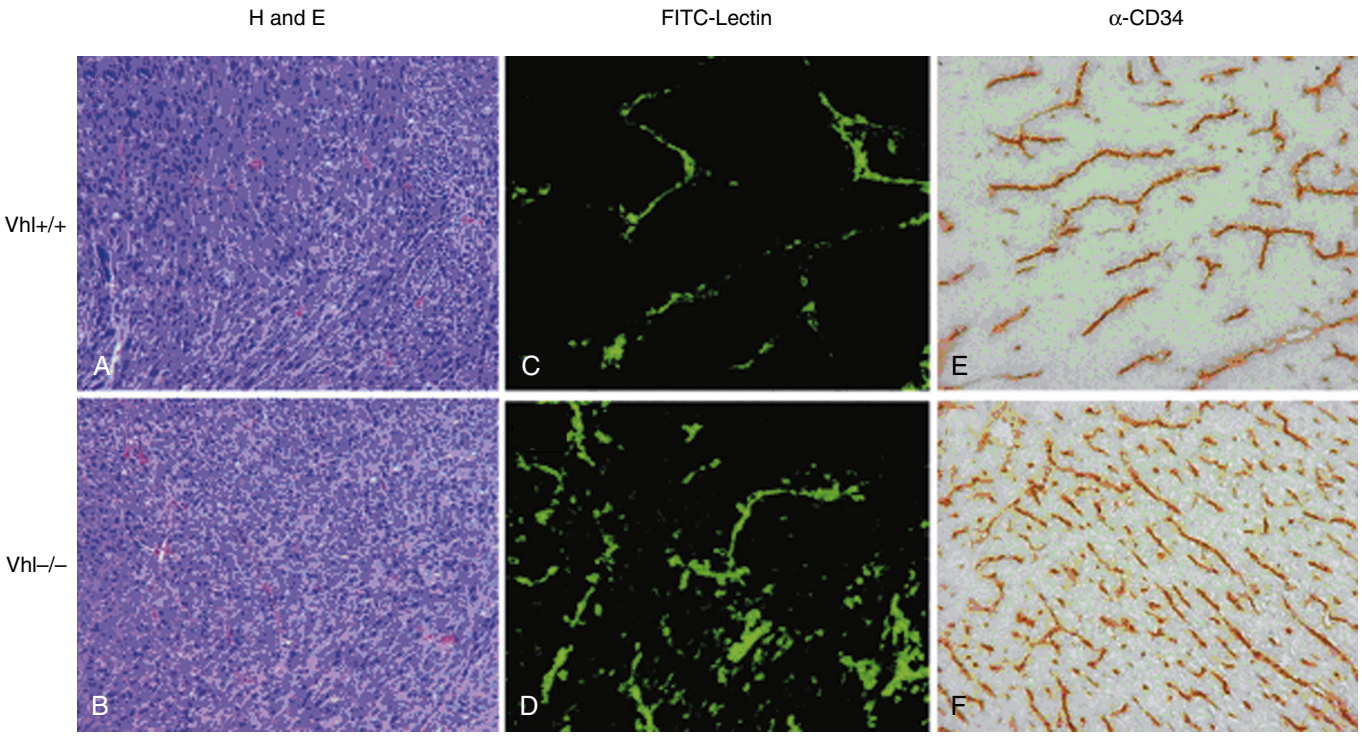


Figure 17-5 **Loss of the pVHL tumor suppressor increases tumor angiogenesis** Fibrosarcomas were generated subcutaneously in immunocompromised mice by injecting Ras-transformed fibroblasts derived from wild-type *($Vhl^{+/+}$)* or pVHL-deficient *($Vhl^{-/-}$)* mice. Tumor sections reveal that loss of pVHL, and consequent constitutive HIF activation, correlated with increased tumor angiogenesis. Tumor vessels were labeled with either FITC-lectin **(C, D)** or CD34 antibodies **(E, F).** *H and E,* Hematoxylin and eosin **(A, B).** *(Courtesy F. Mack and M. C. Simon).*

loss of VEGF expression[39] associated with fundamental defects in angiogenesis.[40] The close link between the HIFs and VEGF expression in tumors has prompted the design of specific HIF inhibitors, partly to limit expression of VEGF and other hypoxically induced angiogenic factors in cancer and other diseases.[41,42]

Each of the several VEGF homologs in mammals (VEGF-B, VEGF-C, VEGF-D, and PlGF) has distinct influences on angiogenesis and binds to one or more of the family of VEGF receptors. VEGF-C and VEGF-D regulate lymphangiogenesis through their effects on VEGFR-3/Flt-3, which is expressed on lymphatic ECs.[9] PlGF binds to both VEGFR-1/Flt-1 and the neuropilins, displacing VEGF and thereby making it available for binding to VEGFR-2.[32] Some data, however, suggest that heterodimers of PlGF and VEGF may be more potent in some contexts than the more typical VEGF homodimer.[14] Much work remains to be done to tease apart the unique and overlapping functions of the various VEGF homologs and their receptors.

bFGF

Basic FGF (bFGF or FGF2) is one of more than 20 known fibroblast growth factors that mediate a large number of developmental and homeostatic functions in different tissues. bFGF was identified biochemically in a search for angiogenic molecules released by tumor cells. When added to tissues exogenously or overexpressed in transplanted tumor cells, bFGF has potent angiogenic properties.[43] Like VEGF, bFGF binds to extracellular heparan sulfate proteoglycans and activates cognate receptor tyrosine kinases. Interestingly, loss-of-function studies have failed to reveal an inherent role of bFGF in embryonic angiogenesis, although this may be due to functional complementation by other FGF family members. As many of the angiogenic properties of bFGF appear to require VEGF function, however, one important role of bFGF in tumor angiogenesis may be to induce VEGF expression.[43] The situation is almost certainly more complex, because VEGF and bFGF act synergistically in some contexts but clearly have independent effects on ECs in others. The emerging picture suggests that bFGF and many other angiogenic factors act as general growth and survival factors for ECs partly by regulating VEGF expression, whereas VEGF itself may preferentially stimulate many of the cellular processes that lead to new vessel formation.

Angiopoietins/Tie Receptors

In addition to VEGF and FGF receptors, ECs express the Tie1 and Tie2/Tek receptor tyrosine kinases. Genetic ablation of either Tie1 or Tie2 in mice produced embryos in which vasculogenesis was intact, but subsequent angiogenic remodeling was inhibited. Soluble forms of these receptors were used to identify endogenous ligands, called *angiopoietins* (Ang1-4) (reviewed in Ref. 44). Deletion of Ang1 produced a phenotype similar to loss of Tie2, supporting a role for Ang1 as an important activator of Tie2 signaling. Interestingly, Ang2 also binds to Tie2 with high affinity but does not stimulate Tie2 tyrosine phosphorylation or downstream signaling. Transgenic overexpression of Ang2 produced a phenotype similar to that associated with loss of Ang1 or Tie2, suggesting that Ang2 may be a naturally occurring inhibitor of Ang1 signaling. The role of Ang2 became clearer when it was found to be induced in concert with VEGF at sites of vascular remodeling. Several studies have suggested a model in which Ang2 interferes with the stabilizing effects of Ang1 (such as increased pericyte and smooth muscle recruitment), thereby allowing VEGF to stimulate EC division and migration more efficiently. The roles of Ang3 and Ang4 are less clear, and a cognate ligand for Tie1 has not yet been identified,[44] although Tie1 may act primarily as a repressor of Tie2 signaling.[45]

PDGF

Maturation and maintenance of the vascular system require the establishment of a close functional relationship between ECs and pericytes (PCs). ECs undergoing active division and morphogenesis express PDGF-B, and PCs express the corresponding receptor PDGFRβ. Genetic ablation of either ligand or receptor in mice disrupts PC recruitment, resulting in leaky, malformed blood vessels and increased EC apoptosis.[46] Bergers and colleagues identified a population of c-Kit+, Sca-1+ bone marrow–derived progenitor cells that are recruited to perivascular sites in tumors, where they differentiate into PCs and stabilize the tumor vessels in a PDGFRβ-dependent manner.[47] Overexpression of PDGF promoted recruitment of PCs and tumor vessel stabilization, whereas inhibition of PDGF signaling reduced PC recruitment with a concomitant increase in EC apoptosis.[25] Consequently, a combination of therapies that target both tumor ECs and PCs may prove to be a particularly effective approach.[25,48]

Anti-angiogenic Factors

In his landmark 1971 paper, Judah Folkman not only proposed that tumor growth depends on angiogenesis, but also suggested that endogenous angiogenic inhibitors could be identified and used therapeutically.[4] Intensive efforts over the subsequent three decades have led to the identification of more than 30 endogenous inhibitors whose application can block angiogenesis in a variety of assays and genetic models.[49,50] These naturally occurring compounds include proteolytic cleavage products of extracellular matrix proteins (thrombospondin, endostatin, tumstatin), the protease

plasminogen (angiostatin), and clotting factors (cleaved antithrombin III and prothrombin kringle-2), as well as immune modulators such as interferons and interleukins.[49] The specific function of each of these compounds in tumor angiogenesis, and their possible utility as therapies for cancer treatment, continues to be an area of active investigation.

Thrombospondin 1 (TSP-1)

Initially identified as an extracellular glycoprotein with cell-adhesive properties, TSP-1 binds to integrin and nonintegrin cellular receptors, cytokines, growth factors, and extracellular proteases. TSP-1 is thought to act as a molecular scaffold that facilitates interactions between proteins that regulate cell morphology, signaling, and adhesion, possibly by promoting receptor clustering.[51] In 1990, Bouck, Polverini, and colleagues described the strong anti-angiogenic activity of a TSP-1 proteolytic fragment.[52] Targeted deletion of TSP-1 in mice increased tumor angiogenesis and growth, and subsequent reports confirm the inability of TSP-1 mutant mice to mount a normal angiogenic response in other assays.[53] The *Tsp-1* gene has been shown to be a direct target of the p53 tumor suppressor, and TSP-1 expression has been inversely correlated with the progression of carcinomas and melanoma in humans.[49] The molecular mechanisms by which TSP-1 blocks angiogenesis are likely to be complex, but may include integrin inhibition, interference with VEGF and bFGF signaling, and/or induced expression of the pro-apoptotic FasL protein on ECs.[49] The identification of the gene encoding TSP-1 as a direct p53 target suggests yet another mechanism whereby p53 inactivation can promote tumor progression.

Endostatin and Tumstatin

Both endostatin and tumstatin are proteolytic cleavage fragments derived from collagen molecules. Endostatin was initially purified from a murine hemangioendothelioma cell line and identified as a 20-kDa carboxy-terminal fragment of type XVIII collagen. Recombinant endostatin has multiple anti-angiogenic properties, including the ability to interfere with VEGF and bFGF signaling, inhibit EC motility, and induce EC cell cycle arrest and apoptosis.[49] Endostatin appears to mediate these pleiotropic effects by binding EC integrins, including α5β1, αVβ3, and αVβ1. Tumstatin consists of a 28-kDa fragment of the α3 chain of type IV collagen, promotes EC apoptosis, and suppresses the growth of various human tumor cells in xenograft experiments. Similar to endostatin, tumstatin binds to integrins and thereby inhibits activation of downstream signaling pathways.[49,50] Despite their similarities, endostatin and tumstatin peptides share little sequence identity and can clearly mediate independent functions: For example, endostatin inhibits EC migration with little effect on VEGF-induced proliferation, whereas tumstatin inhibits EC proliferation without significantly affecting migration.

It is interesting to note that many endogenous angiogenesis inhibitors are generated by proteolytic degradation of ECM proteins, or from proteins involved in blood clotting, and that many bind directly to integrin receptors. Growing evidence supports the notion that these compounds play an important role in fine-tuning the angiogenic response that accompanies thrombosis and tissue repair.[54] The production of these endogenous angiogenesis inhibitors may also help explain tumor dormancy, as first proposed by Folkman in 1971. Control of local angiogenic activity in a tumor is thought to be determined by the balance of pro-angiogenic factors (VEGF, angiopoietin 1, bFGF, etc.) and angiogenesis inhibitors (TSP-1, endostatin, tumstatin, etc.). Consequently, it may take months or years to generate the proper genetic and physiological conditions necessary to tip the balance to favor active blood vessel development and tumor growth—an event called the *angiogenic switch*.[55]

Multiple lines of evidence from preclinical models, as well as patient data, support the role of an angiogenic switch in regulating tumor growth. For example, several murine genetic models have shown that tumors generated by transgenic expression of oncogenes initially remain small, with tumor cell proliferation largely offset by apoptosis. After a period of relative stasis, the tumors begin to show evidence of increased vascularity, after which they grow rapidly.[55] The synthesis of angiogenesis inhibitors by a primary tumor may also keep distant metastases from progressing, as removal of a large primary tumor often correlates with the rapid outgrowth of previously unidentified metastatic tumors in patients.[4,50] It has also been suggested that bone marrow–derived EPCs may play an important role in controlling this event and may regulate a critical step in the progression of micrometastases into macrometastases.[16] Collectively, a growing body of data suggests that manipulating the angiogenic switch can control tumor growth; this has prompted the development of clinically relevant angiogenesis inhibitors as cancer therapies.

Targeting Tumor Angiogenesis in Patients

Over the past 15 years, a large number of clinical trials have been conducted to test the efficacy of anti-angiogenic compounds in cancer therapy. Early results, however, were mixed: For example, early trials of endostatin and other compounds that showed promise in preclinical models yielded disappointing results in the clinic,[56,57] although exceptions included a trial showing positive effects of angiostatin in

treating non–small-cell lung cancer when combined with cytotoxic chemotherapy.[58] Some possible reasons for the apparent discrepancies between dramatic results in preclinical models and actual patient responses are discussed next. Despite this somewhat rocky beginning, a number of targeted therapies have shown clinical benefit and have attained approval from the U.S. FDA and the EMA (European Medicines Agency) for treating cancer. In particular, the prominent role of VEGF in tumor angiogenesis made it an obvious therapeutic target, and multiple drugs have been developed that either sequester free VEGF (reducing its effective concentration) or block VEGFR-dependent signaling. The large number of preclinical and clinical studies on drugs directed against VEGF signaling provides an instructive paradigm for the potential successes and shortcomings of other anti-angiogenic strategies to treat cancer.

VEGF-Based Therapies

In 1993, Ferrara and colleagues reported that a murine anti-human VEGF monoclonal antibody could inhibit the growth of different human tumor cell lines in immunocompromised mice, although the antibody had no effect on tumor cell proliferation in vitro.[59] Subsequent analysis revealed that the antibody blocked angiogenic activity in these xenografts, and this led to the development of a humanized version of the antibody, called *bevacizumab* or *Avastin*, for human clinical trials. In 2003, results from two clinical trials of bevacizumab function generated tremendous excitement in the field. In one phase III trial, patients with advanced metastatic colorectal cancer were treated with bevacizumab in conjunction with cytotoxic chemotherapy[60] and displayed an average increase in *overall survival (OS)* of approximately 4 months (from 16 to 20). Although this response seems modest, it was the first indication that specific targeting of VEGF in highly metastatic human cancer could have a survival benefit. In a separate phase II trial, patients with metastatic renal cancer showed a significant, dose-dependent increase in *progression-free survival (PFS)* when treated with bevacizumab compared to placebo.[61] Interestingly, an Fab fragment of bevacizumab (marketed as Lucentis) has shown great success in treating patients with the angiogenic, or "wet," form of age-related macular degeneration.[62]

Since garnering FDA approval as a first-line treatment (along with standard chemotherapy) for metastatic colorectal cancer in 2003, bevacizumab has been subjected to more than 400 different clinical trials in a broad array of cancer types. The FDA approved bevacizumab for treating advanced non-squamous non–small-cell lung cancer (NSCLC) in 2006, metastatic breast cancer in 2008, and metastatic renal cancer and recurrent gliobastoma multiforme in 2009. In each case, increases in PFS were observed, although improved OS was reported in only some cases (e.g., NSCLC patients had increased OS by 2 to 4 months[63]). In 2011, the FDA withdrew its approval for bevacizumab in treating metastatic breast cancer, citing the absence of an OS benefit, and the UK also rejected bevacizumab for this purpose in 2012.

What explains the relatively short-lived responses observed in the clinic, given that bevacizumab clearly increases PFS in multiple cancers, and diverse preclinical models have shown that this activity correlates with predicted reductions in MVD and perfusion? Compensatory signaling through alternative pro-angiogenic pathways (e.g., bFGF) is likely a major factor in acquired resistance to bevacizumab and disease progression, as observed for other selectively targeted therapies. It has also been proposed that decreased perfusion increases tumor hypoxia, which then promotes tumor cell invasion and metastasis promoted in part by HIF activation. Recently, two groups reported increased tumor cell invasiveness and metastasis in independent murine cancer models when mice were treated with a variety of different anti-VEGF therapies.[64,65] Although these results suggest that anti-angiogenics may ultimately drive hypoxic tumor progression, they remain controversial.[35] It is also possible that recruitment of pro-angiogenic EPCs and bone marrow–derived myeloid cells to tumors could help tumors overcome anti-VEGF therapy.[66] In a similar vein, the demonstration that elevated tumor hypoxia can select for the emergence of more malignant tumor cell clones is potentially worrisome.

The relatively short-term improvements attained with bevacizumab and other anti-VEGF therapies may reflect the aggressive and advanced nature of the tumors treated in most clinical trials and suggests that effects on earlier stage tumors might be more dramatic. Surprisingly, a recent randomized phase III trial on patients with stage II and III colon cancer demonstrated no benefit when bevacizumab was added to the standard FOLFOX chemotherapy regimen.[67,68] These data suggest that bevacizumab (and possibly other anti-angiogenic compounds) may not prove helpful in treating early-stage disease, although this needs to be tested directly in multiple tumor types.

Although substantial evidence from preclinical models indicates that bevacizumab blocks tumor angiogenesis, Jain and colleagues have proposed an alternative model in which bevacizumab (and other anti-angiogenic therapies) may actually "normalize" highly aberrant tumor vasculature by restoring the local balance of pro- and anti-angiogenic signaling.[69] Vessel normalization would therefore be predicted to improve overall perfusion and consequent delivery of chemotherapeutic compounds. This model is consistent with the observation that anti-angiogenic therapies are generally not effective as single agents but function best when combined with standard-of-care chemotherapy. If true, it also raises the

specter that anti-angiogenic therapy could paradoxically provide a tumor with a more functional vasculature, ultimately promoting its growth. Recently, however, Van der Velt and colleagues measured perfusion and delivery of radiolabeled chemotherapeutic agents to tumors in NSCLC patients directly and found no evidence that bevacizumab promoted vessel normalization in this setting.[70] In contrast, bevacizumab treatment rapidly decreased tumor perfusion and infiltration of ^{11}C-docetaxel, effects that were maintained for several days posttreatment. Although a preliminary study with only 10 patients, these data argue strongly for similar direct measurement of perfusion and drug delivery in distinct tumor types, as vessel normalization could still be an important phenomenon in other settings.

We have thus far focused primarily on bevacizumab, as it is the most well-studied anti-VEGF therapy in clinical use to date, but there are many other VEGF-related drugs currently in clinical trials. These include small-molecule kinase inhibitors such as *sorafenib* (Bayer-43-0009, Nexavar), originally designed as a Raf kinase inhibitor. Sorafenib also binds and inhibits VEGFR-2/Flk-1, VEGFR-3/Flt-3, and PDGFRβ and was approved by the FDA for treating renal cancer in 2006 and hepatocellular carcinoma in 2007. Another small-molecule kinase inhibitor, *sunitinib* (Sugen11248), that inhibits all three VEGF receptors, c-Kit, and PDGFRβ was approved for treating renal cancer and imatinib-resistant gastrointestinal stromal tumors (GISTs) in 2006. Given the broad substrate specificity of both drugs, however, the degree to which their anti-angiogenic activity underlies clinical effectiveness is not entirely clear. In addition, multiple VEGF-related therapies developed over the past decade, including small-molecule kinase inhibitors and monoclonal antibodies, are in clinical trials for cancer treatment. Similar drugs targeted toward distinct angiogenic signaling pathways are also under development as cancer therapies and will be the focus of additional preclinical and clinical investigation in the coming years.

Metronomic Therapy

Traditional chemotherapy regimens are based on treating patients with drugs at the *MTD* (maximum tolerated dose), typically followed by a break period to allow recovery from the toxic and myelosuppressive effects of the chemotherapeutic agent. MTD chemotherapy typically causes a significant decrease in the number of circulating hematopoietic cells, including neutrophils and other myeloid cells, as well as EPCs. These drops can be quite precipitous and are usually followed by a rapid "rebound" period in which circulating progenitors are mobilized from the bone marrow, a response observed both in mice and in humans. One potentially unfortunate consequence of this response is the increase in EPCs, which, along with recruitment of bone marrow–derived myeloid cells, such as TEMs, could contribute directly or indirectly to tumor angiogenesis (Ref. 71 and references therein). The breaks in MTD regimens may therefore allow repair or expansion of the tumor vasculature and reduce cytotoxic benefit. Although the precise nature and function of EPCs and their differentiated progeny cells remain controversial, there is evidence that VEGF and other angiogenic factors stimulate their release from the bone marrow.[17] Therefore, the addition of anti-angiogenic drugs to standard chemotherapeutic treatments may suppress the ability of tumors to recruit EPCs and their progeny during the drug-free break periods between MTD treatments.

The reduction in drug-free breaks appears to have an additional inhibitory effect on tumor angiogenesis. In 2000, the effects on tumor growth in mice were greater when an MTD regimen was changed to one in which animals were treated with low doses of the same drug but at more frequent intervals.[72] Surprisingly, tumor growth was inhibited or reversed, despite the fact that (in some cases) the tumor cells were themselves resistant to the same cytotoxic drug. These results suggest that the chemotherapy was not only targeting the tumor cells but also inhibiting normal cells such as ECs or recruitment of EPCs. Subsequent work showed that regular, low-dose chemotherapy (also termed *metronomic* dosing) induced the expression of the angiogenic inhibitor TSP-1 in mice, and that genetic deletion of TSP-1 promoted tumor growth and angiogenesis in this model.[53] In fact, one might predict that normal ECs, which are neither transformed nor genetically unstable, would be more sensitive to the cytotoxic effects of chemotherapeutics than tumor cells. By treating patients with a sustained, low dose of the drug and avoiding the breaks inherent to MTD regimens, it is possible that EC recruitment to the tumor vasculature could be more uniformly suppressed, thereby limiting tumor growth.

Remaining Challenges

The clear success of anti-angiogenic therapies, tempered by the relatively short gains in OS, suggests that oncologists will have a new and growing arsenal of weapons to complement standard chemo- and radiation-based therapies in the future. As always, caution is necessary, as preclinical data have only rarely predicted the outcome of treatments in patients. One reason for this discrepancy is that many preclinical studies have continued to rely on xenograft models, in which a large number of highly malignant tumor cells are introduced subcutaneously into recipient mice. Although a quick and

reproducible approach, it is perhaps not surprising that events that are rate limiting for xenograft growth may have little to do with those controlling human cancer progression. Some anti-angiogenic compounds, such as endostatin, profoundly limited or regressed tumor growth in xenografts but failed to show any significant benefit in early clinical trials.[56,57] The development of genetically altered strains of mice that more closely mimic the development and histology of human cancers[73] may offer more predictive preclinical models for anti-angiogenic therapy.

It is increasingly clear that anti-angiogenic therapies are likely to be most effective when combined with other treatments, for the reasons elaborated earlier. Currently, however, it is essentially impossible to predict which specific combinations of drugs, and which specific delivery strategies, are likely to be effective in inhibiting angiogenesis for a given tumor type or in a given patient.[74] The largely encouraging clinical trial results argue for additional research to determine precisely how tumor and stromal cells respond to specific targeted anti-angiogenic therapies, as well as metronomic therapy. Equally important, protocols need to be developed to integrate anti-angiogenic approaches effectively with standard MTD chemotherapy and radiation therapy. Finally, tumors can develop resistance to specific angiogenesis inhibitors, either by altering the expression of multiple pro- and anti-angiogenic factors or by modulating the tumor stroma. A great deal more research will be necessary to establish even the most general guidelines, but the potential benefits of treating cancer patients with angiogenesis inhibitors are considerable. It is likely that our current understanding of tumor angiogenesis, and our ability to manipulate it clinically, will have once again altered greatly by the next edition of this book.

Key References

7. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438:937-945.
8. Domigan CK, Iruela-Arispe ML. Recent advances in vascular development. *Curr Opin Hematol*. 2012;19:176-183.
9. Saharinen P, Eklund L, Pulkki K, et al. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. *Trends Mol Med*. 2011;17:347-362.
10. Christiansen A, Detmar M. Lymphangiogenesis and cancer. *Genes Cancer*. 2011;2:1146-1158.
11. Fantin A, Vieira JM, Gestri G, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*. 2010;116:829-840.
12. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141:39-51.
13. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer*. 2004;4:437-447.
14. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology*. 2005;69(suppl 3):4-10.
15. Bertolini F, Shaked Y, Mancuso P, et al. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer*. 2006;6:835-845.
16. Li Calzi S, Neu MB, Shaw LC, et al. EPCs and pathological angiogenesis: when good cells go bad. *Microvasc Res*. 2010;79:207-216.
17. Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell*. 2006;124:175-189.
18. Kopp HG, Ramos CA, Rafii S. Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr Opin Hematol*. 2006;13:175-181.
19. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. 2005;8:211-226.
20. Lewis CE, De Palma M, Naldini L. Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. *Cancer Res*. 2007;67:8429-8432.
21. Neri D, Bicknell R. Tumour vascular targeting. *Nat Rev Cancer*. 2005;5:436-446.
23. Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. *Genes Cancer*. 2011;2:1117-1133.
25. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005;438:932-936.
26. Folkman J. Endogenous angiogenesis inhibitors. *APMIS*. 2004;112:496-507.
27. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005;438:967-974.
30. Olsson AK, Dimberg A, Kreuger J, et al. VEGF receptor signalling—in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7:359-371.
32. Guttmann-Raviv N, Kessler O, Shraga-Heled N, et al. The neuropilins and their role in tumorigenesis and tumor progression. *Cancer Lett*. 2006;231:1-11.
33. Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30:393-402.
34. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer*. 2012;12:9-22.
35. De Bock K, Mazzone M, Carmeliet P. Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? *Nat Rev Clin Oncol*. 2011;8:393-404.
40. Ramirez-Bergeron DL, Runge A, Adelman DM, et al. HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. *Dev Cell*. 2006;11:81-92.
41. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. *Nat Rev Drug Discov*. 2003;2:803-811.
42. Semenza GL. Development of novel therapeutic strategies that target HIF-1. *Expert Opin Ther Targets*. 2006;10:267-280.
43. Presta M, Dell'Era P, Mitola S, et al. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. 2005;16:159-178.
44. Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. *Exp Cell Res*. 2006;312:630-641.
45. Singh H, Tahir TA, Alawo DO, et al. Molecular control of angiopoietin signalling. *Biochem Soc Trans*. 2011;39:1592-1596.
46. Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine Growth Factor Rev*. 2004;15:215-228.
47. Song S, Ewald AJ, Stallcup W, et al. PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat Cell Biol*. 2005;7:870-879.
48. Holash J, Thurston G, Rudge JS, et al. Inhibitors of growth factor receptors, signaling pathways and angiogenesis as therapeutic molecular agents. *Cancer Metastasis Rev*. 2006;25:243-252.
49. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res*. 2005;65:3967-3979.
50. Folkman J. Angiogenesis. *Annu Rev Med*. 2006;57:1-18.
51. Kazerounian S, Yee KO, Lawler J. Thrombospondins in cancer. *Cell Mol Life Sci*. 2008;65:700-712.
53. Bocci G, Francia G, Man S, et al. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci U S A*. 2003;100:12917-12922.
54. Serini G, Valdembri D, Bussolino F. Integrins and angiogenesis: a sticky business. *Exp Cell Res*. 2006;312:651-658.

57. Sridhar SS, Shepherd FA. Targeting angiogenesis: a review of angiogenesis inhibitors in the treatment of lung cancer. *Lung Cancer*. 2003;42(suppl 1): S81-S91.
58. Kurup A, Lin CW, Murry DJ, et al. Recombinant human angiostatin (rhAngiostatin) in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer: a phase II study from Indiana University. *Ann Oncol*. 2006;17:97-103.
60. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 2004;350:2335-2342.
61. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med*. 2003;349:427-434.
62. Lim LS, Mitchell P, Seddon JM, et al. Age-related macular degeneration. *Lancet*. 2012;379:1728-1738.
63. Sandomenico C, Costanzo R, Carillio G, et al. Bevacizumab in non small cell lung cancer: development, current status and issues. *Curr Med Chem*. 2012;19:961-971.
64. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 2009;15:220-231.
65. Ebos JM, Lee CR, Cruz-Munoz W, et al. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell*. 2009;15:232-239.
66. Mancuso P, Bertolini F. Circulating endothelial cells as biomarkers in clinical oncology. *Microvasc Res*. 2010;79:224-228.
67. Sargent DJ. Chemotherapy: failure of bevacizumab in early-stage colon cancer. *Nat Rev Clin Oncol*. 2011;8:10-11.
68. Van Cutsem E, Lambrechts D, Prenen H, et al. Lessons from the adjuvant bevacizumab trial on colon cancer: what next? *J Clin Oncol*. 2011;29:1-4.
69. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov*. 2011;10:417-427.
70. Van der Veldt AA, Lubberink M, Bahce I, et al. Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. *Cancer Cell*. 2012;21:82-91.
71. Kerbel RS. Antiangiogenic therapy: a universal chemosensitization strategy for cancer? *Science*. 2006;312:1171-1175.
72. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer*. 2002;2:727-739.
73. Gutmann DH, Hunter-Schaedle K, Shannon KM. Harnessing preclinical mouse models to inform human clinical cancer trials. *J Clin Invest*. 2006;116:847-852.
74. Dalton WS, Friend SH. Cancer biomarkers—an invitation to the table. *Science*. 2006;312:1165-1168.

References

1. Ferrara N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer*. 2002;2:795-803.
2. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539-545.
3. Algire GH, Chalkley HW, Earle WE, et al. Vascular reactions of normal and malignant tissues in vivo. III. Vascular reactions of mice to fibroblasts treated in vitro with methylcholanthrene. *J Natl Cancer Inst*.. 1950;11:555-580.
4. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
5. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407:249-257.
6. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671-674.
7. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438:937-945.
8. Domigan CK, Iruela-Arispe ML. Recent advances in vascular development. *Curr Opin Hematol*. 2012;19:176-183.
9. Saharinen P, Eklund L, Pulkki K, et al. VEGF and angiopoietin signaling in tumor angiogenesis and metastasis. *Trends Mol Med*. 2011;17:347-362.
10. Christiansen A, Detmar M. Lymphangiogenesis and cancer. *Genes Cancer*. 2011;2:1146-1158.
11. Fantin A, Vieira JM, Gestri G, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*. 2010;116:829-840.
12. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141:39-51.
13. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer*. 2004;4:437-447.
14. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology*. 2005;69(suppl 3):4-10.
15. Bertolini F, Shaked Y, Mancuso P, et al. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat Rev Cancer*. 2006;6:835-845.
16. Li Calzi S, Neu MB, Shaw LC, et al. EPCs and pathological angiogenesis: when good cells go bad. *Microvasc Res*. 2010;79:207-216.
17. Grunewald M, Avraham I, Dor Y, et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell*. 2006;124:175-189.
18. Kopp HG, Ramos CA, Rafii S. Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr Opin Hematol*. 2006;13:175-181.
19. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. 2005;8:211-226.
20. Lewis CE, De Palma M, Naldini L. Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. *Cancer Res*. 2007;67:8429-8432.
21. Neri D, Bicknell R. Tumour vascular targeting. *Nat Rev Cancer*. 2005;5:436-446.
22. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*. 1996;379:88-91.
23. Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. *Genes Cancer*. 2011;2:1117-1133.
24. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst*. 2002;94:883-893.
25. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005;438:932-936.
26. Folkman J. Endogenous angiogenesis inhibitors. *APMIS*. 2004;112:496-507.
27. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005;438:967-974.
28. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435-439.
29. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. 1996;380:439-442.
30. Olsson AK, Dimberg A, Kreuger J, et al. VEGF receptor signalling—in control of vascular function. *Nat Rev Mol Cell Biol*. 2006;7:359-371.
31. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376:62-66.
32. Guttmann-Raviv N, Kessler O, Shraga-Heled N, et al. The neuropilins and their role in tumorigenesis and tumor progression. *Cancer Lett*. 2006;231:1-11.
33. Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30:393-402.
34. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer*. 2012;12:9-22.
35. De Bock K, Mazzone M, Carmeliet P. Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? *Nat Rev Clin Oncol*. 2011;8:393-404.
36. Kotch LE, Iyer NV, Laughner E, et al. Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol*. 1999;209:254-267.
37. Peng J, Zhang L, Drysdale L, et al. The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. *Proc Natl Acad Sci U S A*. 2000;97:8386-8391.
38. Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J*. 1998;17:3005-3015.
39. Maltepe E, Schmidt JV, Baunoch D, et al. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature*. 1997;386:403-407.
40. Ramirez-Bergeron DL, Runge A, Adelman DM, et al. HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. *Dev Cell*. 2006;11:81-92.
41. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. *Nat Rev Drug Discov*. 2003;2:803-811.
42. Semenza GL. Development of novel therapeutic strategies that target HIF-1. *Expert Opin Ther Targets*. 2006;10:267-280.
43. Presta M, Dell'Era P, Mitola S, et al. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev*. 2005;16:159-178.
44. Eklund L, Olsen BR. Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling. *Exp Cell Res*. 2006;312:630-641.
45. Singh H, Tahir TA, Alawo DO, et al. Molecular control of angiopoietin signalling. *Biochem Soc Trans*. 2011;39:1592-1596.
46. Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine Growth Factor Rev*. 2004;15:215-228.
47. Song S, Ewald AJ, Stallcup W, et al. PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat Cell Biol*. 2005;7:870-879.
48. Holash J, Thurston G, Rudge JS, et al. Inhibitors of growth factor receptors, signaling pathways and angiogenesis as therapeutic molecular agents. *Cancer Metastasis Rev*. 2006;25:243-252.
49. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res*. 2005;65:3967-3979.
50. Folkman J. Angiogenesis. *Annu Rev Med*. 2006;57:1-18.
51. Kazerounian S, Yee KO, Lawler J. Thrombospondins in cancer. *Cell Mol Life Sci*. 2008;65:700-712.
52. Good DJ, Polverini PJ, Rastinejad F, et al. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A*. 1990;87:6624-6628.
53. Bocci G, Francia G, Man S, et al. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci U S A*. 2003;100:12917-12922.
54. Serini G, Valdembri D, Bussolino F. Integrins and angiogenesis: a sticky business. *Exp Cell Res*. 2006;312:651-658.
55. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86:353-364.
56. Herbst RS, Hess KR, Tran HT, et al. Phase I study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol*. 2002;20:3792-3803.
57. Sridhar SS, Shepherd FA. Targeting angiogenesis: a review of angiogenesis inhibitors in the treatment of lung cancer. *Lung Cancer*. 2003;42(suppl 1): S81-S91.
58. Kurup A, Lin CW, Murry DJ, et al. Recombinant human angiostatin (rhAngiostatin) in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer: a phase II study from Indiana University. *Ann Oncol*. 2006;17:97-103.

59. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature*. 1993;362:841-844.
60. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 2004;350:2335-2342.
61. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med*. 2003;349:427-434.
62. Lim LS, Mitchell P, Seddon JM, et al. Age-related macular degeneration. *Lancet*. 2012;379:1728-1738.
63. Sandomenico C, Costanzo R, Carillio G, et al. Bevacizumab in non small cell lung cancer: development, current status and issues. *Curr Med Chem*. 2012;19:961-971.
64. Paez-Ribes M, Allen E, Hudock J, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*. 2009;15:220-231.
65. Ebos JM, Lee CR, Cruz-Munoz W, et al. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell*. 2009;15:232-239.
66. Mancuso P, Bertolini F. Circulating endothelial cells as biomarkers in clinical oncology. *Microvasc Res*. 2010;79:224-228.
67. Sargent DJ. Chemotherapy: failure of bevacizumab in early-stage colon cancer. *Nat Rev Clin Oncol*. 2011;8:10-11.
68. Van Cutsem E, Lambrechts D, Prenen H, et al. Lessons from the adjuvant bevacizumab trial on colon cancer: what next? *J Clin Oncol*. 2011;29:1-4.
69. Carmeliet P, Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov*. 2011;10:417-427.
70. Van der Veldt AA, Lubberink M, Bahce I, et al. Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. *Cancer Cell*. 2012;21:82-91.
71. Kerbel RS. Antiangiogenic therapy: a universal chemosensitization strategy for cancer? *Science*. 2006;312:1171-1175.
72. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer*. 2002;2:727-739.
73. Gutmann DH, Hunter-Schaedle K, Shannon KM. Harnessing preclinical mouse models to inform human clinical cancer trials. *J Clin Invest*. 2006;116:847-852.
74. Dalton WS, Friend SH. Cancer biomarkers—an invitation to the table. *Science*. 2006;312:1165-1168.

Swarnali Acharyya, Lynn Matrisian, Danny R. Welch, and Joan Massagué

18 Invasion and Metastasis

In the written history of medicine, neoplasms have been diagnosed for nearly 4000 years. Almost from the beginning, medical practitioners recognized that the most life-threatening attribute of neoplastic cells is the ability to disseminate and colonize distant tissues. When tumors are diagnosed and have not spread beyond the tissue of origin, cure rates for most cancers approach 100%. However, when tumor cells have established colonies elsewhere, cancer is often incurable.

The process of converting a normal cell into a life-threatening metastatic cancer cell is referred to as tumor progression (Figure 18-1). As discussed in previous chapters, medicine has evolved toward a recognition that neoplasia is a cellular disease, and further advanced to understand the molecular underpinnings of the early stages of progression resulting in cancer development. It is now recognized that metastases represent a subset of cells that have left the primary tumor, which are behaviorally distinct from the cells remaining at the site of tumor origin, and the molecular mechanisms underlying the phenotypic differences that characterize a metastatic cell are being elucidated.

Generation of a Metastatic Cell

Metastasis is defined as the dissemination of neoplastic cells to discontiguous nearby or distant secondary sites where they proliferate to form a mass. But how did tumor cells acquire the ability to metastasize? The answer to this question requires examination of the mechanisms underlying how tumors arose and progressed toward increasingly aggressive behavior.

By the time a neoplasm is diagnosed, it comprises at least 10^9 cells. Yet, even cursory examination of a tumor histologically reveals that the cells are pleiomorphic. Furthermore, if one isolates single cell clones from a tumor, they vary dramatically in terms of biological behavior.

Tumor heterogeneity exists for virtually every phenotype measured.[1,2] There are three types of heterogeneity within a tumor: positional, temporal, and genetic. Positional heterogeneity is determined by the accessibility of a cell to external stimuli (e.g., oxygen [O_2] levels). For example, radiation sensitivity is proportional to oxygenation; therefore, two identical cells would exhibit differences in radioresponse depending on distance from a capillary. Temporal heterogeneity is relevant with regard to changes in cells due to cyclical signals. Cells in the G_0/G_1 phase of the cell cycle would be less sensitive than cells in the S phase to drugs targeting DNA replication. Genetic heterogeneity is the result of inherent properties of tumor cells themselves. Isolation of single-cell clones confirms that there are inherent differences between subpopulations comprising a single tumor mass.

The heterogeneity of tumors raises an important question regarding tumor origin: Are tumors of unicellular or multicellular origin? Tumors express maternal or paternal isoenzymes, but rarely both, strongly suggesting that they arose from a single cell. Analysis of karyotypes reveals that virtually all cells within a tumor share a common abnormal chromosomal change (e.g., all CML cells have t[9;22]). Additional karyotypic abnormalities may be superimposed on the shared ones. If tumors are monoclonal, how, then, does heterogeneity arise?

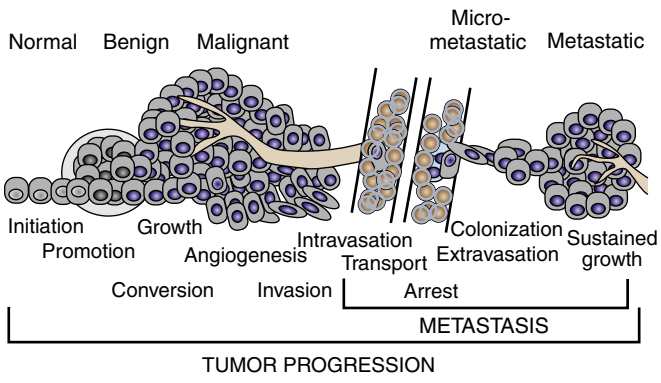


Figure 18-1 Tumor progression

The generation of heterogeneity requires divergence of single transformed cells into multiple phenotypically distinct progeny. The process appears to be fundamental to tumor progression, but it also occurs in normal physiology. For example, pluripotent hematopoietic stem cells can generate cells along multiple lineages, and a single fertilized egg yields a multicellular organism with organs and tissues. Although stem cell theory accommodates diversification, the molecular mechanisms underlying differentiation and diversification of both normal and cancer cells are still being elucidated (see Chapter 10). The journey of a metastatic cell is described in the next sections.

Tumor Invasion

Tumor invasion, the capacity for tumor cells to disrupt the basement membrane and penetrate underlying stroma, is the distinguishing feature of malignancy. Invasion requires major changes in cell morphology and phenotype, in particular for epithelial cells that represent the precursors to over 90% of human cancers. Normal epithelial cells form polarized sheets maintained by tight junctions, adherens junctions that organize the actin (microfilament) and tubulin (microtubule) cytoskeleton, and desmosomes attached to keratin-containing intermediate filaments. They are anchored to the basement membrane by hemidesmosomes and their associated intermediate filaments and integrin contacts that organize actin. Invasion requires alterations in cell-cell and cell-matrix adhesion, coordinated with matrix degradation and cellular motility (Figure 18-2).[5] The structural and regulatory proteins that control cell adhesion and migration are key downstream targets of oncogene and tumor suppressor-controlled signaling pathways, providing insights into how oncogenic transformation results in progression to an invasive phenotype. An interesting observation has been that many of the molecules implicated in tumor invasion also affect other processes involved in tumor progression, including cell survival, growth, apoptosis, and angiogenesis, highlighting the intricacy of the network of interrelated pathways that controls cellular behavior.[6]

Adhesion

Invasion of epithelial cell–derived carcinomas often involves dramatic changes in cell shape. Conversion from an epithelial morphology to a nonpolarized, motile, spindle-shaped cell resembling a fibroblast is referred to as the *epithelial-mesenchymal transition* (EMT).[7] EMT is characterized by the loss of epithelial-specific E-cadherin from the adherens junctions and a switch from the expression of keratins as the major intermediate filament to the mesenchymal intermediate-filament vimentin. EMT is not cancer specific; it is a normal process that occurs during embryonic development and wound healing. EMT is influenced by the tumor microenvironment and is observed primarily at the edge of the tumor in contact with tumor stroma. Soluble factors, in particular transforming growth factor-β and hepatocyte growth factor/scatter factor, are regulators of EMT. Tumor cells may reverse the process and undergo a mesenchymal-epithelial transition (MET) in the absence of EMT-inducing signals. The transient nature of EMT helps explain why metastatic cells can morphologically resemble cells in the primary tumor despite the fact that they by necessity have accomplished all the steps of the metastatic cascade.

Epithelial cell-cell interactions are mediated primarily by cadherins, transmembrane glycoproteins that form calcium-dependent homotypic complexes. The epithelial-specific cadherin, E-cadherin, functions as a tumor suppressor and a metastasis suppressor.[8] Loss of E-cadherin correlates with increased invasion and metastatic potential in most tumor types. Reexpression of E-cadherin in experimental models can block invasion, suggesting that E-cadherin loss is indeed causative. Loss of E-cadherin in cancer occurs through several mechanisms, including transcriptional repression and proteolytic degradation. The zinc finger transcriptional repressors Snail and Slug, in particular, have been implicated in regulating EMT by virtue of their ability to repress E-cadherin transcription. Cadherins are regulated by catenins (α-, β-, γ-, and p120 catenins), cytoplasmic proteins that functionally link the cadherin complex to the actin cytoskeleton. β-Catenin is a cell adhesion protein and a transcription factor. In addition to its role in adherens junctions, it participates in canonical Wnt signaling, a signaling

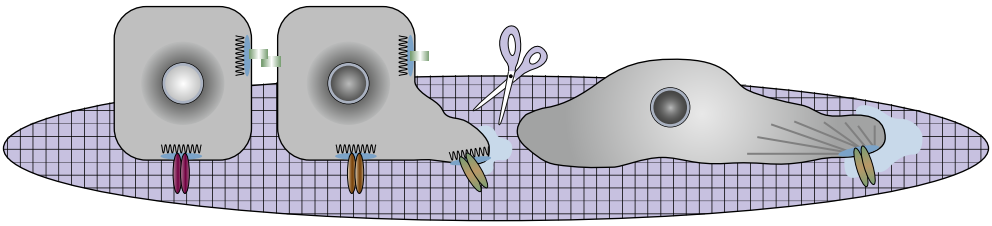


Figure 18-2 The steps of tumor invasion Tumor invasion involves the loss of cell-cell adhesions (cadherins represented by *green bars*), alterations in cell-matrix adhesion (integrins represented by *ovals*), proteolysis of the extracellular matrix (*blue matrix*, degradation demonstrated by clearing of matrix mediated by proteinases represented by scissors), and motility involving alterations in the actin cytoskeleton (*intracellular black* and *gray lines*).

pathway important in development and cancer. E-cadherin levels and function are also disrupted by loss of p120 catenin, which occurs in many tumor types and may also contribute to tumor metastasis.

Loss of function of cell-cell adhesion molecules other than E-cadherin is associated with the ability of tumor cells to invade and metastasize. Neural cell adhesion molecule (NCAM), a member of the immunoglobulin-like cell adhesion molecule Ig-CAM family, is downregulated in several tumor types, and NCAM loss results in an increased ability of tumor cells to disseminate.[8] Other Ig-CAMs, such as DCC (deleted in colorectal carcinoma), CEACAM1 (carcinoembryonic antigen CAM1), and Mel-CAM (melanoma-CAM), also demonstrate reduced expression in specific cancer types. However, not all cell-cell adhesion molecules can be viewed as potential invasion suppressors. N-cadherin promotes motility in some cell types, and Ig-CAMs such as L1, CEA (carcinoembryonic antigen), and ALCAM (activated leukocyte CAM) are often overexpressed in advanced cancers and have functions associated with cancer progression. This complexity may be explained by signaling functions for these molecules, either direct or indirect, that are distinct from their role in cell-cell adhesion. The interrelatedness of tumor growth and tumor invasion, and limitations of experimental model systems, often does not allow a distinction between growth effects that influence the appearance of an invasive phenotype and an effect on cellular invasion per se.

The extracellular matrix (ECM) provides a scaffold for the organization of cells and spatial cues that dictate cell behavior.[9] The ECM is composed of proteins, primarily triple-helical collagens, glycoproteins such as laminins and fibronectin, and proteoglycans. The basement membrane is an organized ECM that separates polarized epithelial, endothelial, and muscle cells from the underlying tissue. Interstitial matrix provides the structure characteristic of connective tissues. The molecular composition of the ECM varies between tissues and organs and provides contextual information to cellular constituents. In addition, the ECM serves as a repository for secreted regulatory proteins and growth factors. Finally, ECM proteins themselves can be active signaling molecules, activities that frequently are only revealed after proteolysis reveals cryptic sites. Thus, the interaction of cells with ECM molecules determines their capacity for survival, growth, differentiation, and migration.

Cells adhere to ECM via integrins, a family of transmembrane glycoproteins assembled as specific combinations of 18 α and 8 β subunits.[5] Integrins bind to distinct but overlapping subsets of ECM components. During tumor progression, cancer cells tend to undergo a switch in their integrin expression pattern, downregulating the integrins that mediate adhesion and maintain a quiescent, differentiated state, and expressing integrins that promotes survival, migration, and proliferation.[10] Although there is a cell-type dependency on integrin function, in general integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are viewed as suppressors of tumor progression, whereas $\alpha_v\beta_3$, $\alpha\beta_6$, and $\alpha_6\beta_4$ promote cellular proliferation and migration. Integrins mediate both "outside-in" and "inside-out" signaling, so that changes in cellular adhesion can alter cellular phenotype, and changes in intracellular signaling pathways can modulate cellular adhesion. A well-described and important mechanism whereby integrin-ECM interactions modulate cell function is by cooperative signaling with different growth factor receptors. Many of the cellular responses induced by activation of tyrosine kinase growth factor receptors are dependent on the cells being able to adhere to an ECM substrate in an integrin-dependent fashion. Signaling in response to ECM ligation usually activates focal adhesion kinase (FAK) and nonreceptor tyrosine kinases of the *src* family.

Matrix Degradation

Disruption of basement membrane is a hallmark of malignancy. Degradative enzymes produced by the tumor cells, and by resident and infiltrating cells as a response to the tumor, contribute to matrix degradation and facilitate tumor cell invasion. Proteolytic enzymes of many classes have been implicated in tumor cell invasion, including the serine proteinases plasmin, plasminogen activator, seprase, hepsin, several kallikreins, the cysteine proteinase cathepsin-B, the aspartyl proteinase cathepsin-D, and metal-dependent proteinases of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) families. Other matrix-degrading enzymes such as heparanase, which cleaves heparin sulfate proteoglycans, and hyaluronidase cleavage of its substrate hyaluronic acid have also been causally associated with tumor progression and invasion.

Liotta and colleagues observed that metastatic potential correlates with the degradation of type IV basement membrane collagen and focused attention on the metal-dependent gelatinases.[11] These enzymes are now recognized as MMP2 and MMP9, and many of the 23 members of the MMP family of matrix-degrading metalloproteinases have been associated with tumor progression. Elevated MMP levels correlate with invasion, metastasis, and poor prognosis in many cancer types, and animal models provide evidence for a causal role for MMP activity in cancer progression.[12] The plasminogen activator/plasmin system has also been causally implicated in cancer invasion,[13] and urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1

(PAI-1) are validated prognostic and predictive markers for breast cancer.[14]

The regulation of matrix proteolysis is complex and can involve the concerted action of multiple proteinases and proteinase classes from both tumor cells and adjacent resident and infiltrating cells (Figure 18-3). The conversion of pro-MMP2 to active MMP2 requires membrane-type MT1-MMP (MMP14), a transmembrane MMP that is activated intracellularly by the proprotein convertase family member, furin. There is evidence for a cascade of cathepsin-D–cathepsin-B–uPA–plasmin–MMP activation that results in activated enzymes capable of degrading all components of the ECM. Proteolysis is also regulated by the production of specific endogenous protease inhibitors, including the tissue inhibitors of metalloproteinases (TIMPs), serine proteinase inhibitors (serpins), and cysteine protease inhibitors (cystatins). These inhibitory activities are produced and secreted by tumor or stromal cell types, and some proteinase inhibitors are stored in high concentrations in the ECM. Proteinase activity cascades can function via proteolytic degradation of some of these proteinase inhibitors in addition to activation of other proteinases.

The original view that proteolytic enzymes function predominantly to remove physical ECM barriers has been expanded with the realization that proteolysis is a key regulator of multiple steps of tumor progression. For example, MMP substrates in the matrix or on the cell surface that modulate cellular growth, differentiation, apoptosis, angiogenesis, chemotaxis, and migration have been identified.[12] The abundant evidence for a role for MMPs in tumor progression led to the design and testing of synthetic MMP inhibitors for cancer therapy.[15] These inhibitors proved to be ineffective in clinical trials, results that have been explained by problems with inhibitor or clinical trial design and a lack of understanding of the broad range of MMP activities resulting in both cancer-promoting and cancer-inhibitory effects.

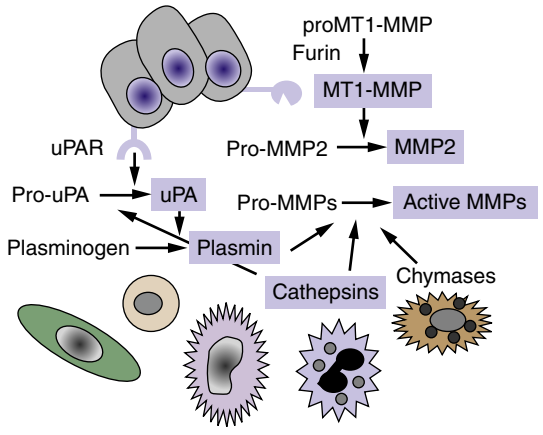


Figure 18-3 Proteolytic cascades Extracellular proteinases are made by tumor cells as well as by stromal fibroblasts and inflammatory cells. Proteolytic cascades result in the conversion of proenzymes to their active form. Enzymes in blue boxes are capable of degrading components of the extracellular matrix (ECM). In many cases, proteolytic cascades are localized to the surface of tumor cells. The urinary plasminogen activator receptor (uPAR) is expressed by many tumor cells and initiates and localizes the conversion of pro-urokinase plasminogen activator (pro-uPA) to its active form, which then converts the serum protein plasminogen into the active serine proteinase, plasmin. The membrane type 1-matrix metalloproteinase (MT1-MMP) is a transmembrane protein that is activated intracellularly by the proprotein convertase furin. MT1-MMP converts pro-MMP2 to its active form, MMP-2. Enzymes of many classes convert pro-MMPs to their active form.

Motility

Cellular locomotion occurs as the result of coordinated polymerization and depolymerization of the actin cytoskeleton to extend a pseudopod at the leading edge of the cell, followed by contraction associated with disassembly of cell-matrix adhesive contacts at the trailing edge.[16] Lamellipodial protrusions at the leading edge are nucleated by a branched actin network involving the Arp2/3 complex and its regulators, the WASp (Wiskott-Aldrich syndrome protein) family, cortactin, and the GTPase Rac. Actin contractility is regulated by myosin light-chain kinase and upstream small GTPases, in particular Rho and its effector Rho-kinase (ROCK). Single cells migrate with a spindle-shaped morphology, referred to as *mesenchymal migration*, or with the less adhesive ellipsoid shape used by leukocytes and *Dictyostelium* termed *amoeboid migration* (Figure 18-4). Collective migration can occur when the cells retain cell-cell junctions and clusters of cells move in single file through a tissue.

Tumor cells can secrete factors that stimulate motility in an autocrine fashion. Tumor cell–produced lysophospholipase D (autotaxin) stimulates motility, as does lysophosphatidic acid (LPA), which can be produced by lysophospholipase D

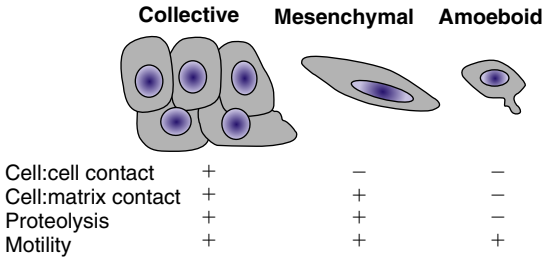


Figure 18-4 Types of cellular invasion Cells can move through matrix barriers as collectives, in which multiple cells remain attached and move together, or as single cells with mesenchymal or amoeboid characteristics. Epithelial-derived tumor cells undergoing collective migration retain cell-cell adhesions, whereas those undergoing mesenchymal or amoeboid movement have reduced or absent cadherin-mediated adhesions. Mesenchymal motility requires proteolysis and integrin-mediated cell-matrix adhesion. In the absence of proteolysis and extracellular matrix (ECM) adhesions, tumor cells can move through ECM using amoeboid movement, similar to that displayed by infiltrating leukocytes. Amoeboid movement is characterized by elevated actin cytoskeleton activity mediated by the small GTPase Rho and its regulator Rho-kinase.

activity on lysophosphatidylcholine. Hepatocyte growth factor/scatter factor (HGF/SF) interacts with its receptor, *c-met*, to induce chemokinetic activity of epithelial cells, resulting in an invasive phenotype. Directional motility is a chemotactic or haptotactic effect in response to a gradient of soluble or localized factors, respectively. Chemotaxis is often the result of growth factors such as IGF, and chemokines of the CCR and CXC family. Haptotaxis is characterized as a response to gradients of ECM components such as laminin-5 and fibronectin and can be modulated positively or negatively by proteolysis.

Coordination of Cancer Invasion

The coordination of cell-cell and cell-matrix adhesion, matrix degradation, and cytoskeletal activity is required for cellular invasion. The type of cell migration (i.e., collective, mesenchymal, or amoeboid) is influenced by the relative levels of adhesion mediated by cadherins and integrins, proteolytic activity, and actin contractility. Modulation of any of these factors can convert one type of motility into another.[16]

Invadopodia is the name that has been given to structures identified in invading cells that represent the physical convergence of the adhesive, proteolytic, and motility components of invasion (Figure 18-5).[4] Invadopodia are actin-rich organelles that protrude from the plasma membrane and contact and locally degrade the ECM. Invadopodia contain adhesion molecules, including several β1 integrins and CD44, the serine proteinases seprase and dipeptidyl dipeptidase IV, and several MMP and ADAM metalloproteinases. Inside the plasma membrane, invadopodia contain actin and actin assembly molecules and multiple signaling molecules including focal adhesion kinase (FAK), *src*-associated proteins such as p130Cas and Tks5/FISH (tyrosine kinase substrate 5/five SH3 domains), and the small GTPases cdc42, Arf1, and Arf6. Thus, invadopodia are implicated as key cellular structures that are used to coordinate and regulate the various components of the process of cancer invasion.

The Metastatic Cascade

Although invasion is required for metastasis, the ability to invade is not sufficient for metastasis (see Figure 18-1). Some tumors are highly aggressive, forming secondary lesions with high frequency (e.g., small-cell carcinoma of the lung,

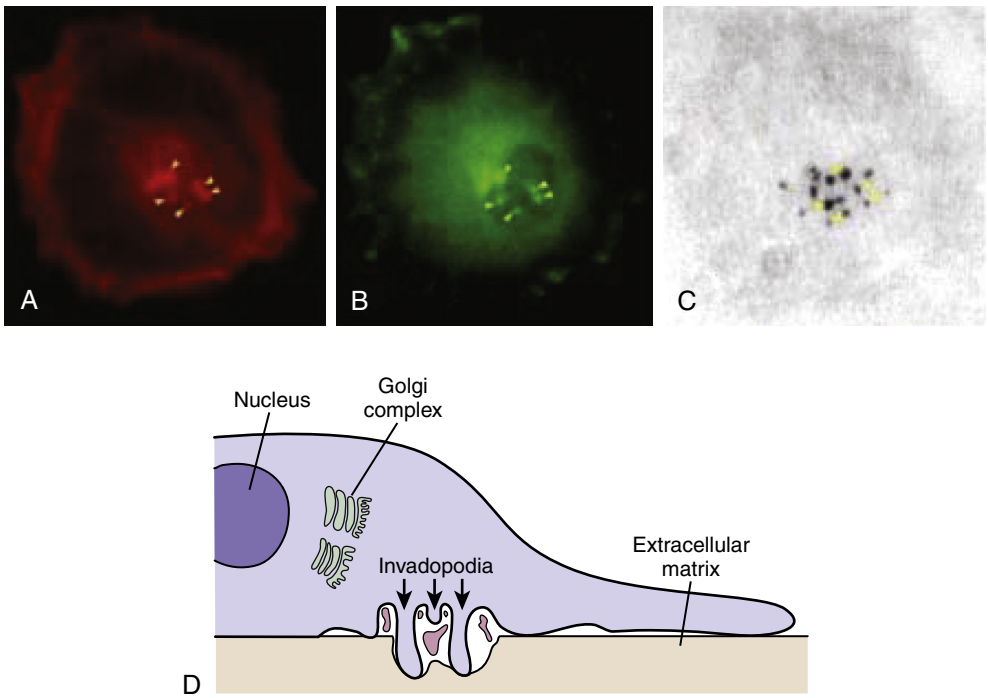


Figure 18-5 Invadopodia Confocal laser image showing triple immunofluorescence labeling of A375MM melanoma cells plated on tetramethylrhodamine isothiocyanate (TRITCJ)-conjugated gelatin. **(A)** Invadopodial structures marked by actin-binding phalloidin-Alexa 546. **(B)** Invadopodial structures marked by Alexa 633-conjugated anti-phosphotyrosine antibodies. **(C)** Degradation areas on the underlying Alexa 488-conjugated gelatin. Image shows colocalization between actin, phosphotyrosine, and patches of degraded extracellular matrix, fulfilling the criteria for the definition of invadopodia. **(D)** Schematic diagram of the invadopodial complex based on correlative light-electron microscopy reconstructions. Spatial relationships with the nucleus and the Golgi complex are shown. Invadopodial protrusions originate from profound invaginations of the ventral surface of the plasma membrane; within the area delimited by the large invagination, large fragments of gelatin can often be seen. *From Ayala I, Baldassarre M, Caldieri G, et al. Invadopodia: a guided tour.* Eur J Cell Biol. *2006;85:159-164, with permission.*

melanoma, pancreatic carcinoma), whereas others are rarely metastatic despite being locally invasive (e.g., basal cell carcinomas of the skin, glioblastoma multiforme). Fidler and colleagues have proposed an analogy regarding metastasis that is highly illustrative. Metastatic cells are likened to athletes participating in the decathlon. Each cell must be capable of completing every step of the metastatic cascade. If a cell cannot complete any step, it cannot go on to subsequent steps and cannot form a metastasis.

Metastasis is primarily thought of as developing via dissemination in the bloodstream, although other routes of spread occur. Carcinoma cells tend to escape and spread initially to draining lymph nodes, becoming trapped and proliferating. The thoracic duct links the lymphatic system to the bloodstream, connecting lymphatic to hematogenous spread. Metastases can also develop by spreading across body cavities. For example, ovarian carcinoma cells most frequently establish secondary tumors by dissemination in the peritoneum while rarely forming metastases via hematogenous spread. Other routes of spread also exist but are far less common (e.g., dissemination of melanoma cells along the space between endothelium and basement membrane or perineural spread in pancreatic and prostatic carcinomas). Thus, the route of dissemination is not inherent to a definition of metastasis.

Intravasation

How tumor cells enter the bloodstream is not clearly understood. The growth of a tumor exerts a hydrostatic pressure, and studies imply that tumor-cell invasive cords follow lines of least resistance. Angiogenesis is likely to be a prerequisite for metastasis, but this has not been formally proven (see Chapter 17). Tumor cell entry into intact blood vessels is an active process that requires serine and metalloproteinase activity in an experimental model of intravasation.[17] Tumor blood vessels, however, are highly abnormal, with fewer pericytes and increased permeability compared with normal vessels, and presumably provide an easier route for direct entry into the bloodstream.[18] Lymphatic vessels are also abnormal, but their role in intravasation is unknown. Regardless of the route, tumor cells enter the circulation in great numbers: Estimates are 3 to 4 million cells/day/g of tumor.[19] The number of tumor cells in the peripheral blood, however, does not necessarily predict whether the patient will develop metastases.[20] In contrast, the detection of disseminated tumor cells in lymph nodes and bone marrow does correlate with metastatic relapse, suggesting that, at least in breast cancer, the properties that allow the cells to find their way to these tissues and survive are the same properties that permit distant metastases.

Transport

Once tumor cells enter a circulatory compartment, they can move actively by motility mechanisms or passively, carried or pushed along with fluid flow. Injection of radiolabeled cells directly into circulation reveals that a substantial proportion is lost during the transport phase of the metastatic cascade. Many tumor cells are eliminated by natural killer (NK) cells or monocytes before arrival in a secondary site. Tumor cells that escape immune recognition are frequently killed by exposure to hemostatic shear forces.[21] Bioassays in the lungs, liver, heart, and muscle have been performed following intravenous injection of tumor cells. It is noted that by the time it takes to remove the tissues for assay (2 to 3 minutes), most cells are dead due to mechanical trauma.[21] The average tumor cell diameter ranges from 20 to 30 μm but must navigate through vessels significantly smaller (e.g., 6- to 7-μm capillaries). Even if tumor cells have the ability to deform and squeeze through the passages, they are subjected to significant hydrostatic pressures. Depending on the tumor type and biophysical parameters such as membrane fluidity, cellular elasticity, and cytoskeletal organization, the cells will remain intact or be broken by shear. Deformability is also affected by the pressures found within various tissues. In contrast to the shear forces usually encountered in the vasculature, blood flow in bone sinusoids is sluggish (about 30-fold less than in capillaries and postcapillary venules), and diameter is not a concern.[22]

During transport, the behavior of tumor cells is often determined by their presence as single cells or as emboli. Embolization can be homotypic (tumor cell–tumor cell) or heterotypic (tumor cell–leukocyte, tumor cell–platelet, tumor cell–fibrin). The association of tumor cells with blood cells can be the result of altered cell surface glycosylation and expression of sialyl Lewis X/A on the tumor cell that permits interaction with a class of vascular adhesion molecules found on normal leukocytes and endothelium, the selectins. Alterations in the adherence of tumor cells to endothelium via E-selectin, platelets via P-selectin, and leukocytes via L-selectin alter the metastatic potential in animal models.[23] Embolus size can also contribute to protection of the tumor cells from biophysical forces or immune attack. In essence, encapsulation of tumor cells helps to protect them. As a result of the consequence of embolus formation, heparin, an inhibitor of selectin/glycan interactions, has been considered as an antimetastatic agent.

Visualization of tumor cells in the circulation during transport indicates that the cells roll rather than float in a manner analogous to leukocytes. Nonetheless, during this time, tumor cells are weakly adherent and subject to anoikis, a specialized type of apoptosis in which cells that are

anchorage dependent are induced to die.[24] In general, metastatic cells are more resistant to anoikis than nonmetastatic cells and are frequently referred to as being anchorage independent. This is somewhat misleading, because some tumor cells will induce apoptosis even if firmly attached to a substrate if that substrate is not the preferred one for the type of cell. It is possible, then, that circulating tumor cells receive sufficient signals from the ECM, other cells, and/or serum proteins to limit their susceptibility to anoikis.

Arrest

It is important to discriminate between the physical trapping and arrest of circulating cells in the microvasculature and selective adhesion to the walls of the microvasculature. Both processes have been observed, and the relative importance of these mechanisms in specific organs is debated.

There are three types of endothelial structures found in higher vertebrates: continuous, discontinuous, and fenestrated. Most endothelial cells form tight junctions with their neighbors and have a continuous, unbroken basement membrane beneath them. However, in certain organs, such as liver and spleen, the endothelial cells and the basement membrane have gaps, or discontinuities, in their structure. In the kidney, a fenestrated endothelium, there are gaps between endothelial cells, but a membrane-like structure connects them and the entire structure overlaps in a continuous basement membrane. The structure of these endothelial/basement membrane barriers contributes to the normal function of the tissues and forms different barriers through which tumor cells must pass.

Adhesion of circulating tumor cells to organ microvessel endothelial cells represents one of the more important steps in metastasis, especially organ-specific metastasis. In general, higher rates of tumor cell–endothelial adhesion correlate well with metastatic potential. In vivo and in vitro kinetic studies indicate that initial attachment of cancer cells occurs preferentially at endothelial cell junctions.[25] Frequently, tumor cells adhere at sites where inflammation is taking place and is most likely related to alterations in cell surface components of endothelial cells at these sites. Tumor cells use many of the same mechanisms to attach to and traverse endothelium as inflammatory cells, including glycan/selectin interactions.

Once tumor cells bind to the endothelium, they induce the endothelial cells to retract and eventually overlap the tumor cell. During this time, there is no loss of electrical resistance, suggesting that tight junction integrity is maintained. Tumor cells then adhere to subendothelial basement membrane components, and a higher rate of tumor cell adhesion to subendothelial basement membrane correlates with metastatic potential. In the case of HT1080 fibrosarcoma cells, the attachment of circulating tumor cells to the lung vasculature is mediated by tumor $\alpha_3\beta_1$ integrin ligation to laminin-5 in the basement membrane.[26] Patches of exposed basement membrane were found to be preexisting using intravital microscopy techniques in isolated, perfused lungs.

Arrested tumor cells can undergo rapid apoptosis. It is envisioned that in some cases this is the result of the lack of suitable survival signals and the initiation of anoikis. In addition, the attachment of tumor cells to endothelium can release nitric oxide (NO) produced by endothelial nitric oxide synthase.[27] NO can induce apoptosis of tumor cells, indicating an active process that contributes to tumor cell loss and metastatic inefficiency.

Extravasation

Extravasation is the process of tumor cells invading from the interior of a vessel into the organ parenchyma. Extravasation was viewed as a rate-limiting step for metastasis formation, but intravital microscopy studies have indicated that extravasation can be a remarkably efficient process, at least in some situations. For example, 87% of B16F1 murine melanoma cells that were injected through the mesenteric vein into the liver were arrested in the liver 90 minutes after injection, and 83% of the injected cells were found in the liver parenchyma by 3 days, indicating that more than 95% of the arrested cells extravasated.[28] The molecular mechanisms underlying extravasation are viewed as being identical to those involved in invasion, and in vitro assays for extravasation reveal a contribution of cellular adhesion molecules, proteinases, and motility factors.

There is controversy as to whether extravasation is required for the formation of metastases. In the case of some pulmonary metastases, there is evidence that tumor cells can attach to the lung endothelium, survive, and grow intravascularly.[29] Extravasation occurs in this model only when the intravascular foci outgrow the vessel.

Colonization

Colonization, the formation of clusters of tumor cells at ectopic sites, represents a highly inefficient step in the metastatic cascade. In the model of B16F1 cells injected into the liver vasculature, only 2% of the injected cells formed micrometastases, and only 0.02% formed lesions that persisted,

grew progressively, and threatened the life of the animal.[28] The formation of micrometastatic lesions requires that the tumor cell must first survive and then grow in the foreign environment. In some tumor types (i.e., breast and melanoma), metastases can arise decades after the treatment of the primary tumor, indicating that tumor cells can survive in a state of dormancy for long periods. Tumor cells can persist as solitary cells, or they can grow to a size of several hundred cells in which the rate of growth is balanced by the rate of apoptosis. Conversion to a clinically detectable metastatic lesion requires the subsequent initiation of angiogenesis (see Chapter 17). The growth of the cells is dependent on factors, primarily soluble growth factors, present at the site of colonization. Although it is natural to focus on factors that promote the growth of tumor cells in selective sites, there is ample experimental evidence showing that some tissues are hostile to tumor cells.

A tumor cell's ability to establish a metastatic lesion is very much dependent on the microenvironment (see Chapter 16). A prime example of this effect is the role of the "vicious cycle" in the propensity for breast carcinoma to metastasize to bone (Figure 18-6).[30] The mammary carcinoma cells produce parathyroid hormone–related peptide (PTHrP), which during pregnancy would function to release calcium from bone stores. Using the same molecular pathways, tumor cell-produced PTHrP acts on its receptors on osteoblasts to release the tumor necrosis factor-α (TNF-α) family member, receptor activator of nuclear factor-κB ligand (RANKL). RANKL interacts with its receptor RANK on osteoclasts and activates them to degrade mineralized bone.

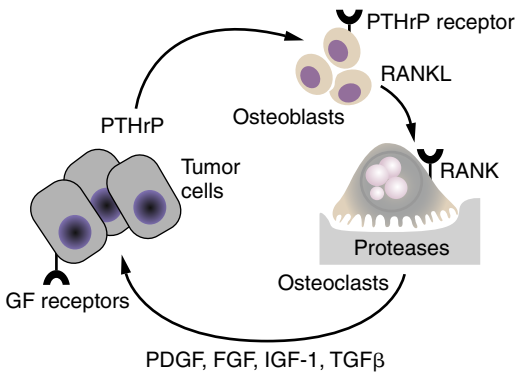


Figure 18-6 The vicious cycle of host-tumor interactions in breast cancer metastasis to bone Breast cancer cells produce parathyroid hormone–related protein (PTHrP), which stimulates bone osteoblasts to express the tumor necrosis factor-α (TNF-α) family member receptor activator of nuclear factor-κB ligand (RANKL). RANKL interacts with its receptor RANK on osteoclast precursors to differentiate into active osteoclasts, resulting in the release of proteases and bone degradation. Growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1), and transforming growth factor-β (TGF-β), which are stored in the bone matrix, are released and stimulate the growth of receptor-containing tumor cells. An increase in tumor cells results in an increase in PTHrP release, leading to a vicious cycle of tumor cell growth and bone degradation.

The bone matrix contains an abundance of growth factors, including PDGF, FGFs, IGF-1, and TGF-β/bone morphogenetic protein family members, which are released during the osteolytic process. It is the release of these growth factors that stimulates the breast cancer cells to grow and to continue to secrete PTHrP and fuel the "vicious cycle." The colonization of breast cancer cells in the bone is thus facilitated by specific characteristics of the bone microenvironment that promote the growth of breast cancer cells.

Organ Selectivity of Metastasis

There is a clear tendency for primary tumors to form metastatic lesions in specific organ sites (Table 18-1). Common regional metastatic involvements can often be attributed to anatomic or mechanical considerations (e.g., efferent venous circulation or lymphatic drainage) and explained by arrest of tumor cells in the first capillary bed or lymph node encountered.[31] Because most tumor cells enter the vasculature in small veins or capillaries, the most common site of metastasis is lung and liver. However, distant metastasis patterns are typically more site specific. In 1889, Paget analyzed postmortem data of women who died of breast cancer and noticed a higher frequency of metastasis to skeleton than would be expected based solely on cardiac output to each organ.[32] He concluded that the pattern of organ distribution of metastases was not simply a matter of chance and suggested that metastases develop only when the "seed" (tumor cells with metastatic ability) and the "soil" (organs or tissues providing growth advantages to seeds) are compatible. Importantly, the mechanical theory and the "seed and soil" hypothesis are not mutually exclusive, and both contribute to metastatic dissemination.

Experimental data supporting the seed and soil hypothesis include preferential invasion and growth of B16 melanoma metastases in specific organs.[33] In addition, palliative treatment of women with advanced ovarian carcinoma has provided an opportunity to test this theory in humans. These patients often have a large ascites burden, but seldom present with disease outside the peritoneal cavity. Tarin and colleagues treated patients with potentially lethal malignant ascites by introducing a tube that drains the peritoneal ascites into the vena cava.[34] In doing so, tumor cells in the ascites were given direct entry into the circulation. Despite continuous entry of billions of viable tumor cells into the circulation, metastases to the lung (i.e., the first capillary bed encountered) were rare. This single clinical observation highlights the inefficiency of the metastatic process and, more important, demonstrates that merely seeding cells in different tissues is not adequate to develop metastases.

Table 18-1 Common Sites of Metastasis

Primary Tumor Site	Most Common Sites of Metastases
Breast	Axillary RLN, contralateral breast via lymphatics, lung, pleura, liver, bone, brain, adrenal, spleen, ovary
Colon	RLN, liver, lung, direct extension into urinary bladder or stomach
Kidney	Lung, liver, bone
Lung	RLN, pleura, diaphragm by direct extension, liver, bone, brain, kidney, adrenal, thyroid, spleen
Ovary	Peritoneum, RLN, lung, liver
Pancreas	Liver, stomach by direct extension, colon, peritoneum
Prostate	Bones of spine and pelvis, RLN
Stomach	RLN, liver, lung, bone
Testis	RLN, lung, liver
Urinary bladder	Direct extension into rectum, colon, prostate, ureter, vagina, bone, RLN, lung, peritoneum, pleura, liver, brain
Uterine endometrium	RLN, lung, liver, ovary

RLN, Regional lymph nodes.

The mechanisms responsible for organ selectivity in tissues can be attributed to the arrest and the colonization steps of the metastatic cascade in particular. Tumor cells adhere more selectively to organ-derived microvascular endothelial cells than to large-vessel endothelial cells, and variants of the B16 melanoma previously selected for metastases to brain, lung, ovary, or liver adhere at a more rapid rate to brain, lung, ovary, or liver endothelial cells, respectively.[35] Using phage-display technology, endothelial cells in different tissues have been demonstrated to express unique markers, and tumor cells recognize the molecular "addresses" to adhere to in a selective manner.[35] Tumor cells are also able to recognize subendothelial basement membrane differences. In vitro studies demonstrate the selective growth of tumor cells in organ-derived soluble growth factors or cells.[36] In vivo, breast tumor cells that express the chemokine receptor CXCR4 preferentially metastasized to tissues that expressed the ligand SDF1/CXCL12.[37] There is a concept that tumor cells colonize in a premetastatic niche initiated in target organs by tumor cell–generated soluble factors that induce the expression of fibronectin by resident fibroblast-like cells.[38] Bone marrow–derived cells that express the vascular endothelial cell growth factor receptor 1 and the integrin $\alpha_4\beta_1$ selectively adhere to these regions, produce the proteinase MMP9 and the chemokine SDF1/CXCL12, and provide a permissive niche for colonization by tumor cells.

Although the data strongly support the notion that there are soluble factors produced in different tissues to which tumor cells can respond, the process of homing has not been validated. Strictly speaking, homing would require directed movement throughout the transit of tumor cells as they leave the primary tumor. Rather, tumor cells distribute according to circulatory patterns initially but may "home" once they are more proximate. Many of the mechanisms used by lymphocytes to home to peripheral lymph nodes or sites of inflammation are apparently shared by tumor cells.

Some of the strongest evidence supporting organ selectivity of cancer cells comes from data showing selection of variants that colonize different tissues. The first selections were done by repetitive isolation of lung metastases from the B16 melanoma followed by reinjection and recolonization.[33] Similar approaches have been used for other tumors, most recently using a human breast carcinoma cell line with selection of metastases to bone, lung, and adrenal gland. Using these breast carcinoma cell lines coupled with a comparison by cDNA microarray has highlighted the requirement for coordinated expression of multiple genes for metastasis.[39] Transcriptomes were compared between parental and bone-selective variants, and over- and underexpressed genes were identified. Among the overexpressed genes in the bone metastasis signature were a matrix metalloproteinase, MMP1; the ECM component osteopontin; the cytokine interleukin-11; the chemokine receptor, CXCR4; and connective tissue–derived growth factor. Subpopulations within the parental population expressed one or more of the bone signature genes, but only a few expressed all of them. Transfection of individual cDNAs only modestly increased bone metastatic efficiency, whereas cotransfection of gene combinations into the parental cells resulted in populations as efficient at bone colonization as the bone-selective variants. Similar studies with a lung-selective variant revealed a lung metastasis signature that overlapped only minimally with the bone metastasis signature.[40] These data highlight that there are specific genes that control metastasis in an organ-specific fashion, and coordinated expression of multiple genes is required.

Metastatic Progression

The journey of a metastatic cancer cell involves several steps from the time it leaves the primary site to the moment it reaches a distant organ. So how long does this journey take to commence, and what is the path that these cells take? Are these metastatic cancer cells or "seeds" disseminated early or late in the life of an evolving primary tumor? These questions are important to understand, because they are linked to the functional consequences of genetic and epigenetic changes that are accrued by metastatic cancer cells when compared to their originating primary tumor. If metastatic progression

occurs early and takes place in parallel to the primary tumor, the genomic landscape of the primary tumor and metastasis can be predicted to be significantly different. On the other hand, if metastatic progression occurs late through clonal evolution within the primary tumor and the cancer cells that left the tumor early became dead ends, then one can expect the primary tumor and metastases to look genetically similar. These questions have direct clinical implications because several drugs that target the primary tumor fail to treat advanced metastatic disease in the clinic. If the metastases were indeed genetically distinct from the primary tumors, it could be envisioned that drugs that target oncogenic changes in the primary tumor would not work for metastases.

Deciphering both the similarities and distinctions between the primary tumor and the metastases may be important in devising strategies to successfully treat metastatic cancer. What is also necessary is gaining an understanding of the regional differences and heterogeneity within the tumor and metastases. A large part of the intratumoral heterogeneity and metastatic diversity is shaped by the distinct tumor microenvironment in which the cancer cells reside. Therefore it can be envisioned that microenvironment-linked selective pressures influence the genetic and epigenetic heterogeneity of tumors and determine the evolutionary trajectory of cancer cells during metastatic progression. Recognition and characterization of such genetic and phenotypic diversity is therefore key for designing rational therapeutic interventions.

The advent of major technological advances over the past decade in the field of sequencing and high-resolution analysis of disseminated cancer cells has made it possible to begin to address such complex biological questions. Deciphering metastatic progression and phylogeny of metastases is an emerging goal as sequencing data from different cancers accumulate, with several hypotheses and models being discussed and debated. Two hypotheses that tower over the rest in explaining the origin of metastatic cells are the linear progression and clonal evolution model on one hand and the parallel progression model on the other. The two models are not mutually exclusive and could be at play to different degrees, depending on the tumor type and its oncogenetic composition.

Linear Progression Model

Pioneering work by the group of Isaiah Fidler has shown that only a subset of preexisting cells within a heterogeneous primary tumor is competent to metastasize.[41] The linear progression paradigm has been the prevalent model to explain this observation. According to this model, cancer cells undergo multiple rounds of mutation and selection in the primary tumor, and only a small subset of these malignant clones acquire the genetic and epigenetic alterations necessary for metastasis.[42,43] A clinical correlation between tumor size and frequency of metastasis is in line with this model.[44] The model also suggests that larger tumors growing over time would have a higher likelihood of containing metastasis-competent clones within their heterogeneous population. The reduced probability of metastasis in cases where primary tumors of less than 2 cm in size are surgically resected in the clinic lends support to this model. New insights into genomic evolution during tumor progression came from Aparicio and colleagues, who sequenced a breast cancer metastasis and its corresponding primary tumor that was removed 9 years earlier.[45] Eleven of the 30 mutations detected in the metastatic lesion were present in the primary tumor. This study revealed that preexisting mutations in the primary tumor do get selected in the metastases, but there is also considerable genomic evolution that occurs during the metastatic process. Comparing the frequency of these somatic mutations in the primary tumors and metastases revealed two discernible trends. Five of the 11 shared mutations were prevalent in the primary tumor, whereas 6 were present in low frequencies. In contrast, all these mutations were prevalent in the metastases, indicating less heterogeneity than the primary tumors. These data are compatible with the clonal expansion model and suggest that a clone from the heterogeneous primary tumor is selected, expands, and generates the distant metastasis. More recently, analysis of tumor evolution using single-cell sequencing technology in two breast cancer cases, by Michael Wigler and colleagues, suggested that a single clonal expansion both formed the primary tumor and seeded the metastasis in the examined cases.[46] It remains to be seen how general these findings are as more matched primary tumors and their metastases in different cancers are sequenced.

In the context of metastasis, what is the time line of metastatic progression? The linear progression model would predict metastatic spread to be a late event in tumor progression, given the time required for the accumulation of sufficient genetic and epigenetic alterations within the primary tumor that permits metastasis. Also implicit in the assumptions from this model and discussed earlier, primary tumors and metastasis are expected to be genetically similar, and a majority of the mutations detected in metastasis should preexist in the primary tumor. Indeed, new insights from genomic sequencing of pancreatic cancer patients suggest that metastasis is a late event in the genetic evolution of pancreatic cancer.[47] Clonal populations that give rise to metastasis preexist within the primary tumor but are more genetically evolved than the original parental, nonmetastatic clone. Using mathematical modeling, Yachida and colleagues showed that a decade passes between the occurrence of an

initiating mutation and the birth of a parental, nonmetastatic cancer cell clone in a pancreatic tumor. Thereafter, at least 5 years pass before metastatic ability is gained in these clones and an average of 2 years elapses until the patient's death. In an analogous manner, colorectal cancer progression was calculated to be 17 years between the birth of an adenoma founder cell and advanced carcinoma; however, only a further 1.8 years pass until the evolution of a metastatic founder cell appears.[48] These studies provide several key insights into the time scale of evolution of metastases in the setting of pancreatic and colon cancer. They suggest that clones in certain advanced cancer already harbor most of the mutations needed for metastatic competence and it takes a relatively short time to develop into metastases. We have to keep in mind that the temporal course of acquisition of metastatic traits might vary greatly between different types of cancers. It is thought that in the case of estrogen receptor–positive breast cancer, there is usually a prolonged latency period, often up to a decade, from gaining infiltration potential and seeding until competence to colonize and outgrow in a distant organ is gained. However, in the case of lung cancer, there is a relatively short interval (often measured in months) between gaining infiltration ability and successful colonization.[49] Large-scale sequencing efforts are under way to reveal the genetic landscape of primary tumors and their metastases in other cancer types, and this could provide insights into the generality of these observations.

Parallel Progression Model

Although there is supportive evidence for the linear progression model, single-cell comparative genomic hybridization analysis of isolated disseminated tumor cells (DTCs) in the bone marrow and primary breast tumors provides an alternative explanation. Christoph Klein and colleagues observed that the genetic changes in the DTCs in the bone marrow did not resemble those in their corresponding primary breast tumor, which led to the conception of the "parallel progression model." According to this model, quasinormal cells disseminate relatively early in the course of tumor progression and evolve independently from the cells in the primary tumor.[50] As a result, genetic and epigenetic evolution occurs through multiple rounds of genetic diversification and clonal selection mostly at the distant organ site(s), which ultimately gives rise to overt metastasis. Parallel evolution thus predicts divergence for many mutations and genetic alterations for the selected cells at the distant sites.[51] Based on the mathematical modeling of tumor growth rates, the parallel progression model questions how large sizes of metastases can be explained by the linear model if they arise only at the advanced stages of tumor progression. Work emerging from several laboratories has shown that tumor cells can indeed disseminate early at the pre-invasive stages in breast cancer progression, both in human breast cancer patients and in experimental mammary tumor models.[52-54] Moreover, Podsypanina and colleagues[54] showed that even untransformed mouse mammary epithelial progenitor cells are able to extravasate and survive at the distant site for prolonged periods and start to grow again on oncogene induction. Collectively, these findings in breast cancer lend support to the parallel progression model and underscore the need for a deeper understanding of metastatic progression in different cancers.

Tumor Self-Seeding

Self-seeding is a recent paradigm that addresses the directionality of tumor seeding by experimental modeling and provides new insights into tumor progression.[55] The concept of self-seeding is not in conflict with either of the models discussed earlier but is complementary to both and provides deeper insights into how the disease can progress. According to this model, cancer dissemination is a bidirectional process in which cancer cells not only seed distant sites but some of these cells reenter the circulation and are attracted back to the original primary tumor.[55] Having egressed to distant organs and having gained increased metastatic abilities, many of these aggressive circulating tumor cells (CTCs) also return to their birthplace using the acquired metastatic abilities. In experimental tumor models, self-seeders are attracted back to the primary tumor by cytokines such as interleukins IL-6 and IL-8. Moreover, seeder cells express high levels of the proteolytic enzyme matrix metalloproteinase 1 (MMP1) and the actin crosslinking protein of invadopodia, Fascin-1, to aid in their infiltration back to the primary tumor. Once back in the primary tumors, self-seeders clonally expand, enriching the tumor population with aggressive clones. Self-seeders may also promote tumor growth using a number of genes, including the chemokine CXC motif ligand1 (CXCL-1) that recruits leukocytes to the tumor microenvironment. Therefore this model predicts that the process of tumor self-seeding can select for aggressive CTCs that can accelerate primary tumor growth and in the process also selects for aggressive subpopulations that are primed for metastasis. Local growth of the primary tumor can therefore be promoted by returning metastatic cells, which in turn can act as a reservoir for breeding aggressive clones through the process of "self-seeding."

In summary, the recent studies on linear progression, parallel progression, and DTC dynamics are clearly bellwethers that have provided new molecular insights into tumor progression. However, it has to be kept in mind that these

efforts represent only early steps of decoding metastatic evolution with several open questions. Though there has been a plethora of supportive evidence for the linear progression model over the past decades, the parallel progression model is intriguing and deserves careful consideration. It is becoming increasingly evident that quasinormal cancer cells can be detected early in circulation during the course of tumor progression, but whether these disseminated cells give rise to pathologically detectable, overt metastasis remains to be shown. None of the models of metastatic progression alone can explain all the phenomena in their entirety in all cancers.

Colonization and Interactions with the Tumor Microenvironment

One of the most challenging and rate-limiting steps of the metastasis cascade is the final step of colonization. On arrival in distant organs, cancer cells need not only to survive in a new and unfamiliar microenvironment but also to grow out into overt metastasis. A large proportion of cancer cells die soon after extravasation; among the few that survive, some remain singly and some in clusters known as *micrometastases*. A vast majority of these cells will stay in a dormant state and never form clinically detectable metastases. However, some of these cells may successfully withstand these unfavorable conditions, or *metastatic stress*, and are able to reinitiate tumor growth, resulting in macrometastases. It is not difficult to envision that the cancer cells that can give rise to metastatic lesions are one of the most aggressively selected populations and are often refractory to most standard therapies.

It is now well recognized that tumors grow in a complex environment composed of multiple cell types and supporting structures together known as the *tumor microenvironment*. Tumor cells interact with their neighbors in this microenvironment, composed of ECM, immune cells, and blood vessels, which influences metastatic success. Recent examples of interactions of metastatic cells with components of the tumor microenvironment are discussed next.

Extracellular Matrix

A major component of the metastasis niche or tumor microenvironment is the ECM, a network of proteins, proteoglycans, glycoproteins, and polysaccharides that constitutes the scaffold and milieu in which all cells sit and migrate. The diverse functions of ECM have been well characterized in primary tumors, where this molecular meshwork provides physical support and serves as a base for cell anchorage. The ECM is also critical in determining polarity and acts as a substrate for migration. Adding to its versatile role, the ECM also acts as a reservoir for bound factors that can be released as bioactive ligands. As a tumor grows with the disruption of tissue organization and remodeling, the mechanical state of the tumor in relation to force, tension, and stiffness of the matrix constantly changes and evolves. ECM remodeling is a highly dynamic process in which the biomechanical cues and ECM alterations influence cell growth, migration, and survival in various stages of tumor progression.[56] For example, lysyl oxidase (LOX) activity is upregulated in several cancers and is tightly linked to the biomechanical properties of the ECM. LOX acts as a crosslinking enzyme that increases ECM stiffness to promote tumor cell invasion and progression in breast cancer models.[57] LOX has been also shown to promote primary tumor growth and metastasis in colon cancer models.[58]

Several studies have shed light on new roles of ECM proteins in premetastatic and metastatic niches. ECM molecules have been shown to be important components of stem cell niches. Perturbation of ECM function by either loss of ECM contact or inhibition of ECM receptor integrins or other ECM binding components leads to reduction of stem cells.[56] Furthermore, ECM proteins can provide anchorage for stem cells that is essential for maintaining their stem-cell characteristics.[59] An interesting example is that of ECM proteins such as fibronectin and osteopontin, which can modulate recruitment of bone marrow–derived cells in the distant microenvironment before the arrival of tumor cells.[60, 61] Such interactions in the premetastatic niche can dictate the pattern and success of colonization of metastatic tumor cells.

The function of ECM proteins as niche components in metastasis is becoming increasingly appreciated. New studies suggest that the ECM provides essential support for metastasis-initiating cells. Recent reports elucidate how two ECM components, tenascin-C (TNC) and periostin, that coexist in the metastatic niche maintain the viability of metastasis-initiating cells. TNC and periostin bind to each other tightly to form scaffolding. TNC enhances metastatic colonization by promoting the survival and fitness of metastasis-initiating cells.[62] Interestingly, stem cell–like breast cancer cells express the ECM component TNC, which enhances their response to Wnt and Notch signaling pathways. TNC expression therefore can help metastasis-initiating cells to thrive in harsh microenvironments and promote metastatic outgrowth. In the lung parenchyma, TGFβ3 stimulates myofibroblast cells that produce the other ECM protein, periostin, which then binds to stromal Wnt factors.[63] Periostin thus concentrates Wnt ligands and presents them to cancer cells, which results in their enhanced ability for lung colonization. Moreover, the cancer cells that benefit from the periostin-Wnt axis are thought to be stem cell–like, metastasis-initiating cells.

Immune Cells

Myeloid cells are the most abundant nucleated hematopoietic cells in the body, consisting of several types of cells with diverse functions. Among myeloid cells, tumor-associated macrophages (TAMs) represent one of the most abundant immune infiltrates in a tumor, and their presence is clinically correlated with poor patient outcome.[64] TAMs belong to a subcategory of macrophages that is associated with immunosuppressive cytokines and pro-angiogenic factors.[65] Several tumor growth-promoting factors and paracrine interactions between cancer cells and macrophages have been characterized in the past few years. Pollard, Condeelis, and colleagues have elucidated how TAMs increase the invasiveness of breast cancer cells via the paracrine interaction between cancer cells and TAMs.[66] Cancer cells express colony-stimulating factor (CSF)1, a critical cytokine for macrophage maturation and activation. CSF1 is also a potent chemoattractant for TAMs that express the CSF1R receptor. TAMs, for their part, express epidermal growth factor that enhances the migration and invasiveness of breast cancer cells expressing the EGF receptor (EGFR). These studies showed how the density of TAMs influences the efficiency of the intravasation of tumor cells. An effector of the CSF1 signaling pathway is the transcription factor Ets2. Deletion of Ets2 in TAMs decreases tumor angiogenesis and lung metastasis in breast tumor models.[67] Yet another interesting interaction is between TAMs and breast cancer cells in the lung involving the cell adhesion molecule vascular cell adhesion molecule 1 (VCAM-1) that promotes cell survival of metastatic cells in the lung.[68] VCAM-1 expressed on breast cancer cells binds to α_4 integrin on TAMs. Thus engaged, VCAM1 activates the adaptor protein Ezrin, which enhances PI3K/Akt signaling in the cancer cells to suppress apoptosis.

Recent studies have shed new light on novel interactions between cells of the adaptive immune system and TAMs that promote metastatic progression. The cross talk between helper T cells and TAMs serves as an example. A large portion of the literature has been focused on T-cell responses in mediating anti-tumor immunity. T-cell activation pathways can be categorized into Th1, Th2, and Th17 responses based on the three different known subsets of helper T cells.[69] Th1 effector cells are characterized by producing γ-interferon, Th2 cells by IL-4 and IL-13 production, and Th17 by IL-17A production. With current advances in understanding the adaptive immune system, it is becoming clear that the presence of $CD8^+$ T cells with markers of Th1 response overall signify good prognosis in solid tumors. Recent studies have revealed that IL-4–expressing $CD4^+$ T cells promote lung metastasis in breast cancer models by affecting macrophage phenotype and effector functions. This prometastatic Th2 CD4 response is mediated by a subset of macrophages that are dependent on IL-4 and IL-13. Such macrophages (referred to as M2 macrophages) produce a number of cytokines, including TGF-β that suppresses antitumor immune responses and EGFR ligands that promote tumor growth. IL-4–producing T cells therefore are able to program macrophages toward a prometastatic phenotype that enhances both tumor invasion and metastasis. Another mechanism by which IL-4 regulates growth-promoting functions of TAM in metastasis is by inducing cathepsin protease activity.[70] Cathepsin proteases are a class of proteolytic enzymes that have been associated with increased motility of tumor cells through the matrix and vasculature. Studies in prostate cancer suggest that T cell and macrophage-derived factor RANKL promotes metastasis.[71] This effect is mediated through the activation of inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK-α). Upon activation, IKK alpha represses a metastasis suppressor gene called *maspin*.[72]

Myeloid-derived suppressor cells, or MDSCs, have become the focus of intense study in cancer biology over the past few years in the context of tumor progression. MDSCs are a heterogeneous population of myeloid cells composed of immature myeloid cells and progenitor cells. These cells are usually not abundant under normal physiological conditions. In healthy individuals, immature myeloid cells differentiate into mature granulocytes or macrophages. However, in individuals with cancer, these bone marrow–derived cells have a partial block in differentiation that results in an expansion of the immature population. The ability of the MDSC population to suppress immune function has major implications for cancer progression. The MDSCs can be categorized into two distinct groups, granulocytic/polymorphonuclear MDSCs (G/PMN-MDSCs) and monocytic MDSCs (M-MDSCs), based on their phenotype.[73] Besides phenotypic differences, the two subsets of MDSCs also differ in the mechanisms they use to suppress immune functions. G-MDSCs use reactive oxygen species (ROS) for immunosuppression, whereas M-MDSCs use inducible nitric oxide synthase (iNOS) and arginase for suppressing immune functions.[74] G/PMN-MDSCs are immature neutrophils that express abnormally high ROS, myeloperoxidase, and lysosomal enzymes compared to differentiated neutrophils (PMN). G/PMN-MDSCs are also less phagocytic than neutrophils. MDSCs isolated from cancer patients with the phenotype $LIN^-HLA^-DR^-CD33^+CD11b^+$ share properties of granulocyte precursors or progranulocytes. The abundance of these cells correlates with poor prognosis and radiographic progression of disease in breast and colorectal cancers. In animal models, MDSCs are shown to infiltrate at the invasive front of tumors, where they contribute to metastasis through enhanced metalloproteinase activity.[75] MDSCs are also responsible for the refractoriness of anti-angiogenic VEGF

inhibitors at least in part by promoting tumor angiogenesis bypassing the VEGF requirement.[76] Recently, G/PMN-MDSCs have been shown to play a critical role in mediating both chemoresistance and metastasis in breast cancer through a network of paracrine signals between carcinoma, endothelial cells, and G/PMN-MDSCs.[77] M-MDSCs in metastatic sites express versican, an ECM proteoglycan that promote metastasis in animal models.[78]

Platelets are specialized blood cells that are produced from megakaryocytes in the bone marrow. Their main function in physiology is to prevent hemorrhage from injury. In the mid-19th century, Trousseau first documented that excessive clotting was related to occult cancer in the body, thus proposing a link between the hemostatic system and malignancy. Since then it has been well recognized that clinical signs of thrombosis or aberrant platelet activation and aggregation are often present in cancer patients with advanced metastatic disease.[79] High platelet counts are associated with poor prognosis in breast, lung, and pancreatic cancers. It has been also shown that depletion of platelets or inhibition of platelet aggregation indeed reduces experimental metastasis in animals.[80] Experimental insights from several laboratories suggest that several functions of platelets might be at play in promoting metastatic progression.[81] Early studies showed that platelets might be functioning in shielding tumor cells from the immune system, in particular from NK cell–mediated tumor lysis.[82] Interestingly, TGF-β released from platelets can also diminish NK function by affecting their granule mobilization and cytotoxicity. Recently Labelle and colleagues showed that platelets could actively signal to tumor cells in transit outside the primary microenvironment.[83] A transient contact between platelets and tumor cells can induce an EMT phenotype by synergistic action of both TGF-β/Smad and NFκB pathways that can increase the invasiveness and metastatic potential in tumor cells. Ablation of TGF-β in platelets or inhibition of NFκB in tumor cells significantly reduces lung metastasis. However, activation of neither the TGF-β/Smad nor the NFκB pathway alone in this context is sufficient to promote lung metastasis. What this study suggests is that tumor cells that have intravasated without losing their epithelial properties might become more mesenchymal and invasive on interactions with platelets in circulation. Although our understanding of platelet function advances, the pro-metastatic functions of platelets in cancer progression remain to be fully explored. It is interesting that platelets can release the neurotransmitter serotonin on activation and thus modulate vascular tone.[84] Future research is necessary to determine how platelets support metastatic growth by altering blood vessel permeability. Given the promising mechanistic studies on platelet function in metastasis, the clinical correlation of platelet activities and cancer, and the potential for antimetastatic therapy, it is imperative to understand platelet involvement in metastatic progression.

In summary, as advances in genomics usher us into an era of personalized diagnostics and treatment development, combinatorial targeting approaches of inhibiting both cancer cell intrinsic and microenvironment-linked pathways are much needed. A deeper understanding of the biology of metastases will be gained by comparing the genetic and phenotypic characteristics of the primary tumor and associated metastases in different cancers and by predicting the course of the development of metastases.

Challenges and Opportunities in Studying the Biology of Disseminated Cancer Cells

New insights into the process of metastasis are pointing to new clinical opportunities for prognosis and intervention and have paved the way for future research. Beyond this, it is becoming increasingly recognized that genetic variations in individual tumors fueled by gene amplification, specific mutations, and single-nucleotide polymorphisms greatly affect the relative efficacy of anticancer treatment. These observations stimulate the consideration of personalized medicine, where treatment would ultimately be determined by each tumor's unique molecular and genomic features. A critical question for oncologists is to define which cancer patients are at a higher risk of metastatic relapse so that treatments can be tailored to those patients most likely to benefit. Research is currently geared toward developing new technologies for detecting cancer cells in the blood (CTCs) and in various organs (DTCs) and to correlate the presence of these cells with the risk of metastatic relapse.

Advanced molecular, cytometric and immunological approaches have improved the ability to detect, monitor, and analyze single DTCs in the bone marrow and CTCs in the blood of cancer patients. Detecting these cells early could provide insights into the biology of metastatic spread and serve as a diagnostic resource for monitoring the efficacy of current cancer therapy. CTC numbers in several cancers have been shown to be prognostic of disease recurrence.[85] For example, CTC numbers before treatment and at the first follow-up visit after initiation of therapy were found to be independently associated with progression-free and overall survival in patients with metastatic breast cancer.[86] Clinical evidence also exists for the association between the presence of DTCs at the time of tumor resection and postoperative metastatic relapse.[87-89] Several new techniques based on cytometric/immunological and molecular approaches have been developed for the detection of CTCs/DTCs over the

past few years. For example, the commercially available CellSearch system (Johnson & Johnson, USA), which consists of an automated enrichment and immunostaining device, was approved by the FDA for the detection of CTCs in patients with breast, colon, and prostate cancer.[90] Other examples include EPISPOT (epithelial immunospot) for the detection of viable DTCs and CTCs and the microfluid platform called the *CTC chip* that captures CTCs from unfractionated blood under controlled laminar flow. Molecular polymerase chain reaction (PCR)-based techniques are also under development. In the future, analysis of CTCs and perhaps DTCs could also be helpful when estimating the efficacy of therapy. However, as is the case with developing new technologies, there are several hurdles to be overcome. At present, a major limitation is that CTCs can be detected in many more patients than ever experience relapse. Other hurdles include tumor heterogeneity, lack of expression of uniform surface markers, limit of detection, and repeated need for bone marrow sampling in cancer patients, to name only a few. Nonetheless, these efforts represent steps in the right direction that could provide critical tools for diagnosis and guiding personalized treatment of metastatic disease in the future.

Key References

4. Ayala I, Baldassarre M, Caldieri G, et al. Invadopodia: a guided tour. *Eur J Cell Biol*. 2006;85:159-164.
8. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer*. 2004;4:118-132.
10. Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol*. 2004;5:816-826.
13. Danø K, Behrendt N, Høyer-Hansen G, et al. Plasminogen activation and cancer. *Thromb Haemost*. 2005;93:676-681.
14. Duffy MJ. The urokinase plasminogen activator system: role in malignancy. *Curr Pharmaceut Biotechnol*. 2004;10:39-49.
16. Wolf K, Friedl P. Molecular mechanisms of cancer cell invasion and plasticity. *Br J Dermatol*. 2006;154(suppl 1):11-15.
20. Pierga JY, Bonneton C, Vincent-Salomon A, et al. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin Cancer Res*. 2004;10:1392-1400.
22. Welch DR, Harms JF, Mastro AM, et al. Breast cancer metastasis to bone: evolving models and research challenges. *J Musculoskelet Neuronal Interact*. 2003;3:30-38.
24. Stupack DG, Teitz T, Potter MD, et al. Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature*. 2006;439:95-99.
25. Nicolson GL. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev*. 1988;7:143-188.
26. Wang H, Fu W, Im JH, et al. Tumor cell alpha3beta1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis 1. *J Cell Biol*. 2004;164:935-941.
27. Qiu H, Orr FW, Jensen D, et al. Arrest of B16 melanoma cells in the mouse pulmonary micro circulation induces endothelial nitric oxide synthase-dependent nitric oxide release that is cytotoxic to the tumor cells. *Am J Pathol*. 2003;162:403-412.
35. Ruoslahti E. Vascular zip codes in angiogenesis and metastasis. *Biochem Soc Trans*. 2004;32:397-402.
38. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-827.
39. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 2003;3:537-549.
40. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;436:518-524.
45. Shah SP, Morin RD, Khattra J, et al. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature*. 2009;461:809-813.
46. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. *Nature*. 2011;472:90-94.
47. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*. 2010;467:1114-1117.
48. Jones S, Chen WD, Parmigiani G, et al. Comparative lesion sequencing provides insights into tumor evolution. *Proc Natl Acad Sci U S A*. 2008;105:4283-4288.
49. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer*. 2009;9:274-284.
50. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. 2009;9:302-312.
51. Schmidt-Kittler O, Ragg T, Daskalakis A, et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proc Natl Acad Sci U S A*. 2003;100:7737-7742.
52. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. 2008;13:58-68.
53. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007;450:1235-1239.
54. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science*. 2008;321:1841-1844.
55. Kim MY, Oskarsson T, Acharyya S, et al. Tumor self-seeding by circulating cancer cells. *Cell*. 2009;139:1315-1326.
56. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol*. 2012;196:395-406.
57. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009;139:891-906.
58. Baker AM, Cox TR, Bird D, et al. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. *J Natl Cancer Inst*. 2011;103:407-424.
59. Li L, Xie T. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol*. 2005;21:605-631.
60. McAllister SS, Gifford AM, Greiner AL, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell*. 2008;133:994-1005.
61. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. 2009;9:285-293.
62. Oskarsson T, Acharyya S, Zhang XH, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. 2011;17:867-874.
63. Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2012;481:85-89.
64. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009;9:239-252.
65. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. *Trends Immunol*. 2012;33:119-126.
66. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 2004;64:7022-7029.
67. Zabuawala T, Taffany DA, Sharma SM, et al. An ets2-driven transcriptional program in tumor-associated macrophages promotes tumor metastasis. *Cancer Res*. 2010;70:1323-1333.
68. Chen Q, Zhang XH, Massagué J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. *123 Cell*. 2011;20:538-549.

69. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21:309-322.
70. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
71. Tan W, Zhang W, Strasner A, et al. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature*. 2011;470:548-553.
72. Luo JL, Tan W, Ricono JM, et al. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature*. 2007;446:690-694.
73. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12:253-268.
74. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur J Immunol*. 2010;40:2969-2975.
75. Yang L, Huang J, Ren X, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 2008;13:23-35.
76. Shojaei F, Wu X, Malik AK, et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol*. 2007;25:911-920.
77. Acharyya S, Oskarsson T, Vanharanta S, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell*. 2012;150:165-178.
78. Gao D, Joshi N, Choi H, et al. Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer Res*. 2012;72:1384-1394.
81. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11:123-134.
83. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell*. 2011;20:576-590.
85. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8:329-340.
86. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351:781-791.
87. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7:834-846.
89. Singletary SE, Greene FL, Sobin LH. Classification of isolated tumor cells: clarification of the 6th edition of the *American Joint Committee on Cancer Staging Manual*. *Cancer*. 2003;98:2740-2741.
90. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol*. 2009;6:339-351.

References

1. Heppner GH, Miller FR. The cellular basis of tumor progression. *Int Rev Cytol*. 1988;177:1-56.
2. Welch DR, Tomasovic SP. Implications of tumor progression on clinical oncology. *Clin Exp Metastasis*. 1985;3:151-158.
3. REFERENCE DELETED IN PROOFS.
4. Ayala I, Baldassarre M, Caldieri G, et al. Invadopodia: a guided tour. *Eur J Cell Biol*. 2006;85:159-164.
5. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002;110:673-687.
6. Chambers AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. *J Natl Cancer Inst*. 1997;89:1260-1270.
7. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2:442-454.
8. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer*. 2004;4:118-132.
9. Boudreau N, Bissell MJ. Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr Opin Cell Biol*. 1998;10:640-646.
10. Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol*. 2004;5:816-826.
11. Liotta LA, Tryggvason K, Garbisa S, et al. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*. 1980;284:67-68.
12. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer*. 2002;2:161-174.
13. Danø K, Behrendt N, Høyer-Hansen G, et al. Plasminogen activation and cancer. *Thromb Haemost*. 2005;93:676-681.
14. Duffy MJ. The urokinase plasminogen activator system: role in malignancy. *Curr Pharmaceut Biotechnol*. 2004;10:39-49.
15. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science*. 2002;295:2387-2392.
16. Wolf K, Friedl P. Molecular mechanisms of cancer cell invasion and plasticity. *Br J Dermatol*. 2006;154(suppl 1):11-15.
17. Kim J, Yu W, Kovalski K, et al. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semi-quantitative PCR-based assay. *Cell*. 1998;94:353-362.
18. Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. *Nat Rev Cancer*. 2002;2:266-276.
19. Butler TP, Gullino PM. Quantitation of cell shedding into efferent blood of mammary adenocarcinoma. *Cancer Res*. 1975;35:512-516.
20. Pierga JY, Bonneton C, Vincent-Salomon A, et al. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin Cancer Res*. 2004;10:1392-1400.
21. Weiss L. Metastatic inefficiency. *Adv Cancer Res*. 1990;54:159-211.
22. Welch DR, Harms JF, Mastro AM, et al. Breast cancer metastasis to bone: evolving models and research challenges. *J Musculoskelet Neuronal Interact*. 2003;3:30-38.
23. Varki NM, Varki A. Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: rationale for clinical studies in humans. *Semin Thromb Hemost*. 2002;28:53-66.
24. Stupack DG, Teitz T, Potter MD, et al. Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature*. 2006;439:95-99.
25. Nicolson GL. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev*. 1988;7:143-188.
26. Wang H, Fu W, Im JH, et al. Tumor cell alpha3beta1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis 1. *J Cell Biol*. 2004;164:935-941.
27. Qiu H, Orr FW, Jensen D, et al. Arrest of B16 melanoma cells in the mouse pulmonary micro circulation induces endothelial nitric oxide synthase-dependent nitric oxide release that is cytotoxic to the tumor cells. *Am J Pathol*. 2003;162:403-412.
28. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer*. 2002;2:563-572.
29. Wong CW, Song C, Grimes MM, et al. Intravascular location of breast cancer cells after spontaneous metastasis to the lung. *Am J Pathol*. 2002;161:749-753.
30. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer*. 2002;2:584-593.
31. Weiss L. Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clin Exp Metastasis*. 1992;10:191-199.
32. Paget S. The distribution of secondary growths in cancer of the breast. *Lancet*. 1889;133:571-573.
33. Fidler IJ. Selection of successive tumour lines for metastasis. *Nat New Biol*. 1973;242:148-149.
34. Tarin D, Price JE, Kettlewell MG, et al. Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. *Cancer Res*. 1984;44:3584-3592.
35. Ruoslahti E. Vascular zip codes in angiogenesis and metastasis. *Biochem Soc Trans*. 2004;32:397-402.
36. Nicolson GL, Dulski KM. Organ specificity of metastatic tumor colonization is related to organ-selective growth properties of malignant cells. *Int J Cancer*. 1986;38:289-294.
37. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001;410:50-56.
38. Kaplan RN, Riba RD, Zacharoulis S, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*. 2005;438:820-827.
39. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 2003;3:537-549.
40. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;436:518-524.
41. Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. *Science*. 1977;197:893-895.
42. Cairns J. Mutation selection and the natural history of cancer. *Nature*. 1975;255:197-200.
43. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61:759-767.
44. Koscielny S, Tubiana M, Le MG, et al. Breast cancer: relationship between the size of the primary tumour and the probability of metastatic dissemination. *Br J Cancer*. 1984;49:709-715.
45. Shah SP, Morin RD, Khattra J, et al. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature*. 2009;461:809-813.
46. Navin N, Kendall J, Troge J, et al. Tumour evolution inferred by single-cell sequencing. *Nature*. 2011;472:90-94.
47. Yachida S, Jones S, Bozic I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*. 2010;467:1114-1117.
48. Jones S, Chen WD, Parmigiani G, et al. Comparative lesion sequencing provides insights into tumor evolution. *Proc Natl Acad Sci U S A*. 2008;105:4283-4288.
49. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer*. 2009;9:274-284.
50. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. 2009;9:302-312.
51. Schmidt-Kittler O, Ragg T, Daskalakis A, et al. From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proc Natl Acad Sci U S A*. 2003;100:7737-7742.
52. Husemann Y, Geigl JB, Schubert F, et al. Systemic spread is an early step in breast cancer. *Cancer Cell*. 2008;13:58-68.
53. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007;450:1235-1239.
54. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science*. 2008;321:1841-1844.
55. Kim MY, Oskarsson T, Acharyya S, et al. Tumor self-seeding by circulating cancer cells. *Cell*. 2009;139:1315-1326.
56. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol*. 2012;196:395-406.
57. Levental KR, Yu H, Kass L, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009;139:891-906.

58. Baker AM, Cox TR, Bird D, et al. The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. *J Natl Cancer Inst*. 2011;103:407-424.
59. Li L, Xie T. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol*. 2005;21:605-631.
60. McAllister SS, Gifford AM, Greiner AL, et al. Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell*. 2008;133:994-1005.
61. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nat Rev Cancer*. 2009;9:285-293.
62. Oskarsson T, Acharyya S, Zhang XH, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nat Med*. 2011;17:867-874.
63. Malanchi I, Santamaria-Martinez A, Susanto E, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2012;481:85-89.
64. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009;9:239-252.
65. Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. *Trends Immunol*. 2012;33:119-126.
66. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 2004;64:7022-7029.
67. Zabuawala T, Taffany DA, Sharma SM, et al. An ets2-driven transcriptional program in tumor-associated macrophages promotes tumor metastasis. *Cancer Res*. 2010;70:1323-1333.
68. Chen Q, Zhang XH, Massagué J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. *123 Cell*. 2011;20:538-549.
69. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21:309-322.
70. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
71. Tan W, Zhang W, Strasner A, et al. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature*. 2011;470:548-553.
72. Luo JL, Tan W, Ricono JM, et al. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature*. 2007;446:690-694.
73. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12:253-268.
74. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. *Eur J Immunol*. 2010;40:2969-2975.
75. Yang L, Huang J, Ren X, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 2008;13:23-35.
76. Shojaei F, Wu X, Malik AK, et al. Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. *Nat Biotechnol*. 2007;25:911-920.
77. Acharyya S, Oskarsson T, Vanharanta S, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell*. 2012;150:165-178.
78. Gao D, Joshi N, Choi H, et al. Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer Res*. 2012;72:1384-1394.
79. Honn KV, Tang DG, Crissman JD. Platelets and cancer metastasis: a causal relationship? *Cancer Metastasis Rev*. 1992;11:325-351.
80. Kim YJ, Borsig L, Varki NM, Varki A. P-selectin deficiency attenuates tumor growth and metastasis. *Proc Natl Acad Sci U S A*. 1998;95:9325-9330.
81. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11:123-134.
82. Nieswandt B, Hafner M, Echtenacher B, Mannel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res*. 1999;59:1295-1300.
83. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell*. 2011;20:576-590.
84. Skolnik G, Bagge U, Blomqvist G, Dahlstrom A, Ahlman H. Involvement of platelet-released 5-HT in tumor cell lodgement. *J Surg Res*. 1985;38:559-567.
85. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8:329-340.
86. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351:781-791.
87. Aguirre-Ghiso JA. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7:834-846.
88. Hermanek P, Sobin LH, Wittekind C. How to improve the present TNM staging system. *Cancer*. 1999;86:2189-2191.
89. Singletary SE, Greene FL, Sobin LH. Classification of isolated tumor cells: clarification of the 6th edition of the *American Joint Committee on Cancer Staging Manual. Cancer*. 2003;98:2740-2741.
90. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol*. 2009;6:339-351.

Daniela F. Quail and Johanna A. Joyce

19

Inflammation and Cancer

Introduction

Adult tissues contain a multitude of cell types that are spatially and functionally coordinated to regulate normal tissue homeostasis. When a tissue becomes injured, for example, from a skin wound, there is a surge of infiltrating cell types and inflammatory responses within the microenvironment that work in concert to heal the injury and restore tissue homeostasis. Interestingly, tumors share many features with injured tissue, as their microenvironment is characterized by various infiltrating immune cell types and chronic inflammation.[1] However, in cancer, the coordinated cell-cell interactions that are critical during normal tissue homeostasis are disrupted, as the tumor acquires the capacity to chronically circumvent normalizing cues from the microenvironment, and in turn, the microenvironment evolves to accommodate the growing tumor.[2] In this chapter, we discuss representative examples of the positive and negative roles that different types of immune cells can play in cancer, to underscore the importance of inflammation in regulating the initiation and progression of this disease. We review the multifaceted role of inflammation in cancer, with a focus on hematopoiesis, chronic injury and tumorigenesis, and the contributions of myeloid and lymphoid cell types to a growing tumor.

Hematopoiesis and the Immune System

Hematopoiesis is the process by which hematopoietic progenitor cells (HPCs) from the bone marrow constitute all mature cell types in the blood (Figure 19-1). As progenitor cells, HPCs have the capacity to either self-renew or differentiate into either myeloid or lymphoid lineages, to ensure that both immature and mature components of the blood system are not depleted.[3] The myeloid lineage of HPC differentiation gives rise to thrombocytes, erythrocytes, mast cells, granulocytes, and monocytes, which further differentiate into macrophages and myeloid dendritic cells. In contrast, the lymphoid lineage of HPC differentiation gives rise to lymphoblasts, which undergo lymphopoiesis to generate B cells, T cells, and natural killer (NK) cells.

There are two arms of the immune system that use cells generated through hematopoiesis to mediate immunogenic functions: the innate and adaptive arms. The innate immune system provides immediate chemical and cellular responses to foreign microorganisms that invade the body. Chemical defenses, including the complement system, consist of biochemical cascades that attack invading cells, via the exponential activation and release of proteases. Cellular defenses are largely mediated by NK and myeloid cell types, including macrophages, dendritic cells (DCs), mast cells, and granulocytes. These cells work together to phagocytose or ingest invading microorganisms, including bacteria and viruses, or to present antigens for recognition by cells of the adaptive immune system. Of note, the adaptive arm of the immune system is largely mediated by lymphoid cell types. Examples include T cells, which recognize antigens presented by major histocompatibility complex (MHC) molecules, and B cells, which recognize antigens in their native form. Whereas the innate immune system provides an immediate defense mechanism against foreign invaders, the adaptive immune system ensures that the body remembers how to protect itself against specific microorganisms in the future.

Chronic Inflammation and Tumor Incidence

The link between chronic inflammation and tumorigenesis was first proposed by Rudolf Virchow in 1863 after he made a seminal observation that linked the presence of infiltrating leukocytes with cancer.[4] Perhaps one of the most straightforward pieces of evidence that deregulated

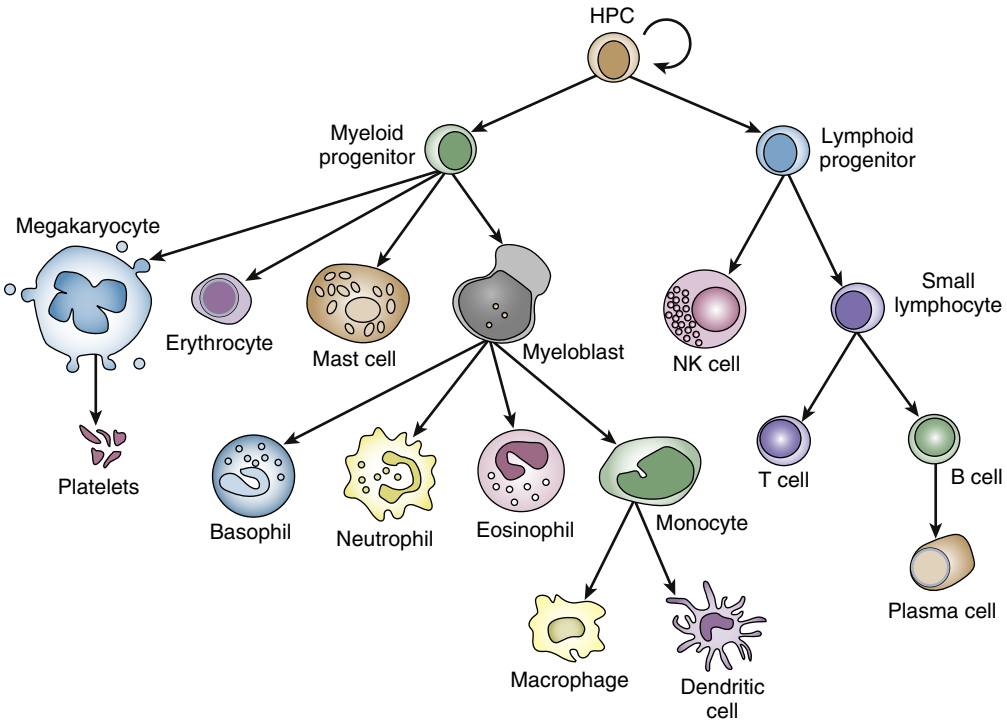


Figure 19-1 Hematopoiesis Hematopoietic progenitor cells (HPCs) can self-renew or differentiate into two multipotent progenitor cell types, myeloid and lymphoid progenitors, which further differentiate to give rise to all cells of the immune system. Myeloid cells differentiate into megakaryocytes, erythrocytes, mast cells, and myeloblasts, which further differentiate into macrophages and dendritic cells. Lymphoid cells give rise to natural killer (NK) cells, and small lymphocytes including T cells (including helper, cytotoxic, and regulatory T cells) and B cells. Together, these cell types work in concert to coordinate both innate and adaptive inflammatory responses.

Table 19-1 Inflammatory Conditions and Infectious Agents That Are Associated with Specific Types of Cancers

Condition/Infection	Associated Neoplasm(s)
Asbestos	Lung cancer
Bronchitis	Lung cancer
Gingivitis	Oral cancer
Inflammatory bowel disease	Colorectal cancer
Skin inflammation (UV)	Skin cancer
Hepatitis	Liver cancer
AIDS	Non-Hodgkin's lymphoma
Chronic pancreatitis	Pancreatic cancer

AIDS, Acquired immunodeficiency syndrome; *UV*, ultraviolet.

inflammation affects tumorigenesis is that tissues which experience chronic injury exhibit a high risk for subsequently developing tumors. Classic examples of tissue damage leading to chronic inflammation include the development of lung cancer arising from tobacco smoke, or skin cancer resulting from exposure to UV (ultraviolet) light (Table 19-1).[1] In both of these cases, the onset of tumorigenesis is supported by a repetitive inflammatory response, whereby immune cells accumulate and their tissue-repair functions become excessive and maladaptive, leading to the development of a pro-tumorigenic niche.[2,5] Recruited inflammatory cells support disease progression by providing critical growth factors and cytokines to sustain tumorigenesis.[5]

Another piece of evidence that deregulated inflammation contributes to tumorigenesis is the correlation between chronic viral infection and cancer initiation. In 1911, the discovery of a tumor virus in chickens by Peyton Rous, later termed the *Rous sarcoma virus* (RSV), was a pivotal discovery in molecular cancer biology that led to the discovery of *src*, the first oncogene.[6,7] Decades later, Bissell and colleagues demonstrated a clear connection between inflammation and tumorigenesis, when they showed that chickens systemically infected with RSV only developed tumors at the site of initial injection or a subsequent inflicted wound.[8,9] It is also known that people infected with hepatitis B or C virus are prone to developing cirrhosis of the liver, which increases the risk of hepatocellular carcinoma by 100-fold.[10] In fact, it has recently been estimated that approximately 2 million cancer cases worldwide, representing 16% of total cases, are caused by infectious agents every year.[11] For a list of infectious agents, inflammatory conditions, and associated cancers, refer to Table 19-1.[1]

Recent attempts to understand the connection between infection, inflammation, and cancer have led to the Human Microbiome Project, which was initiated by the National Institutes of Health (NIH) Roadmap for Medical Research.[12] The project was launched in an effort to gain insight into how microorganisms influence health and disease, given that the human body contains 10 times more microbial cells than human cells, and 100 times more microbial genes (i.e., the microbiome) than human genes.[13] Indeed, it is estimated that in humans, the distal gut contains up to 1000 species and 7000 strains of microbes. It is currently

Table 19-2 Stromal Cell Populations in the Tumor Microenvironment Are Defined by Various Cell Surface Markers and Have Distinct Functions During Tumorigenesis

Cell Type	Functions in the Tumor Microenvironment
Myeloid Lineage	
TAM	Classically activated M1 macrophages are pro-inflammatory and anti-tumorigenic and secrete T_H1 cytokines. Alternatively activated M2 macrophages are anti-inflammatory and pro-tumorigenic and secrete T_H2 cytokines. TAMs exhibit an M2 phenotype; their presence in tumors supports angiogenesis and invasive phenotypes.
TEM	TEMs are monocytes that express the angiopoietin receptor TIE-2. TEMs play a role during tumor angiogenesis through paracrine signaling with angiopoietin-expressing endothelial cells.
Neutrophil	N1 neutrophils are pro-inflammatory and anti-tumorigenic and secrete T_H1 cytokines. N2 neutrophils are anti-inflammatory and pro-tumorigenic and secrete T_H2 cytokines. TGFβ mediates the transition from an N1 to an N2 phenotype.
Mast cell	Mast cells are important in generating and maintaining innate and adaptive immune responses. Mast cells are recruited to tumors where they release factors that enhance proliferation of endothelial cells to promote tumor angiogenesis.
MDSC	MDSCs are elevated in circulation of patients with cancer. Their main function is to disrupt tumor immunosurveillance by interfering with T and NK cell function and promoting M2 macrophage polarization.
Lymphoid Lineage	
NK cell	NK cells are cytotoxic lymphocytes that can kill stressed cells in the absence of antigen presentation. NK cells can detect and kill tumor cells through "missing-self" activation (loss of healthy cell markers) or "stress-induced" activation (gain of stressed cell markers).
CD4+ T_H cell	CD4+ helper T (T_H) cells can be divided into T_H1 and T_H2 lineages. T_H1 cells secrete pro-inflammatory cytokines and are anti-tumorigenic. T_H2 cells secrete anti-inflammatory cytokines and are pro-tumorigenic. The ratio of T_H1:T_H2 cells in cancer correlates with tumor stage and grade.
T_{REG} cell	T_{REG} cells play divergent roles in cancer. They elicit pro-tumorigenic roles by suppressing immunosurveillance; yet their presence in tumors is positively correlated with overall survival in multiple cancer types. These divergent roles may be attributed to context-dependent functions or an inability to distinguish between subpopulations using conventional markers.
CD8+ T_C cell	CD8+ cytotoxic T (T_C) cells are effector cells of the adaptive immune system. They specifically recognize and destroy cancer cells through perforin and granzyme-mediated apoptosis.
B cell	B lymphocytes are important mediators of humoral immunity. In cancer, they play a pro-tumorigenic role by secreting pro-tumorigenic cytokines and altering T_H1:T_H2 ratios. Their importance in supporting tumor growth is evident in B-cell–deficient mice, which exhibit resistance to engraftment of syngeneic tumors.

known that microorganisms contribute to the development of diseases including cancer, despite often maintaining symbiotic relationships with the human body.[13-15] For example, it has been shown that stomach cancer can arise from chronic gastric inflammation caused by *Helicobacter pylori* infection. Inflammatory bowel disease, comprising ulcerative colitis and Crohn's disease, is also associated with recurrent bacterial infection and can predispose to colorectal cancer.[16,17] However, the specific mechanisms and intercellular interactions that disrupt microbial homeostasis, leading to inflammation-induced cancer, remain elusive and an area of active investigation.

Inflammation and the Metastatic Cascade

In each of the cases just described, the onset of tumorigenesis is supported by an unresolved inflammatory response that contributes to a pro-tumorigenic niche, characterized by a plethora of different stromal cell types, growth factors, and cytokines (Figure 19-2).[2,5] For example, studies in breast cancer have shown that the type of inflammatory response is an important predictor of tumor development. In particular, acute inflammation involving cytolytic $CD8^+$ T lymphocytes, $CD4^+$ T helper (T_H1) cells, or classically activated M1 macrophages is generally anti-tumorigenic, whereas chronic inflammation involving B lymphocytes, $CD4^+$ T helper (T_H2) cells, or alternatively activated M2 macrophages is frequently pro-tumorigenic (Table 19-2).[2,18,19] These findings demonstrate a complex relationship between tumor cells and their microenvironment and suggest that the development of a pro-tumorigenic niche is highly dependent on the type of immune response that ensues.

The metastatic cascade begins at the primary tumor site, when tumor cells recruit a vascular supply (angiogenesis), invade through the extracellular matrix (ECM), intravasate into the circulation, disseminate through the body, extravasate at secondary sites, and self-renew to sustain secondary tumor growth. Bone marrow–derived cells have diverse effects on each step of the metastatic cascade and

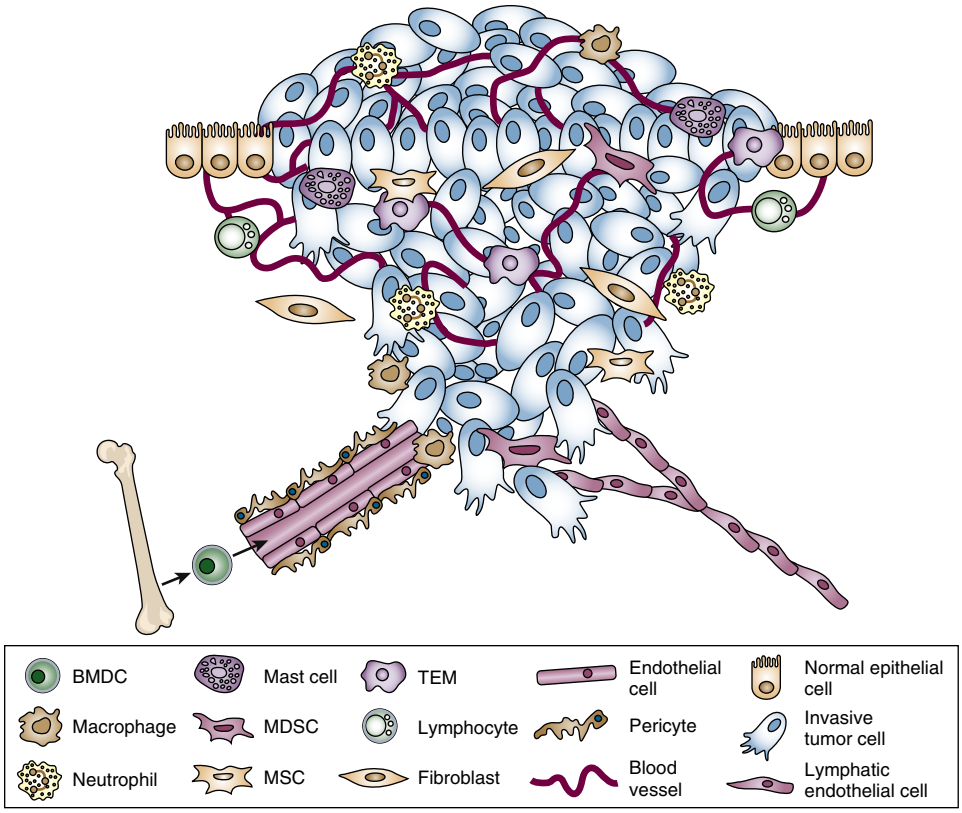


Figure 19-2 The primary tumor environment Cells within a tumor are supported by a complex and dynamic microenvironment composed of multiple infiltrating cell types, including endothelial cells (which line blood and lymphatic vessels), cancer-associated fibroblasts (CAFs), and a variety of bone marrow–derived cells (BMDCs). Infiltrating BMDCs mediate inflammatory responses during cancer progression and can have negative or positive consequences. Major BMDCs within the tumor niche include tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs), mesenchymal stem cells (MSCs), and various other cell types from lymphocyte and monocyte lineages. This tumor-associated cellular cocktail largely dictates the evolution of the surrounding environment and, ultimately, the outcome of disease. *(Image adapted from Joyce JA, Pollard JW. Microenvironmental regulation of metastasis.* Nat Rev Cancer. *2009;9: 239-252).*

render the microenvironment susceptible or resistant to tumorigenic growth.[2] For example, studies in breast cancer have shown that tumor-associated macrophages (TAMs) are critical for promoting angiogenesis and tumor cell invasion and helping cancer cells to cross blood vessel walls. Within the circulation, platelets can enhance the survival of tumor cells by protecting them from NK cell-mediated death and promoting their adhesion to the endothelium at the site of metastasis.[20] Furthermore, myeloid-derived suppressor cells play a role in suppressing immune surveillance of cancer cells, promoting tumor growth.[21] In an elegant study by Lyden and colleagues, hematopoietic progenitor cells (HPCs) positive for vascular endothelial growth factor receptor 1 (VEGFR1) and endothelial progenitor cells positive for VEGFR2 were both shown to be required for mediating neovascularization at sites of future metastasis: the premetastatic niche.[22] Another study reported that recruited $CD11c^+$ DC precursors were capable of assembling tumor-associated neovessels in a model of ovarian carcinoma.[23] As illustrated by these examples, the diverse effects of all these different immune cell types during multiple steps of the metastatic cascade underscore the complexity of tumor-microenvironment relationships in cancer. In the following section, various immune cell types and their roles during tumorigenesis and metastasis are reviewed.

Immune Cells in Cancer

Myeloid Lineage

Macrophages

In normal physiological contexts, macrophages defend against infection, clear debris, and remodel injured tissue to maintain homeostasis. In cancer, normal macrophage function is hijacked by tumor cells to support tumor progression. In fact, in 80% of epithelial cancers, it has been shown that high macrophage infiltration is associated with poor patient prognosis.[24] TAMs typically represent the major immune cell type infiltrating tumors, and in some cancers, such as gliomas and breast cancer, TAMs can constitute up to 30% of the total tumor mass. TAM progenitors are largely recruited from the bone marrow and, once in the tumor mass, represent a critical source of secreted growth factors, proteases, and cytokines that participate in paracrine signaling loops with tumor cells to support invasive phenotypes.[25,26] One important function of TAMs is that they help tumor cells enter blood vessels, a process called *intravasation*. Condeelis and colleagues have published seminal studies using sophisticated multiphoton intravital imaging techniques to observe intimate macrophage–tumor cell interactions during metastatic dissemination in

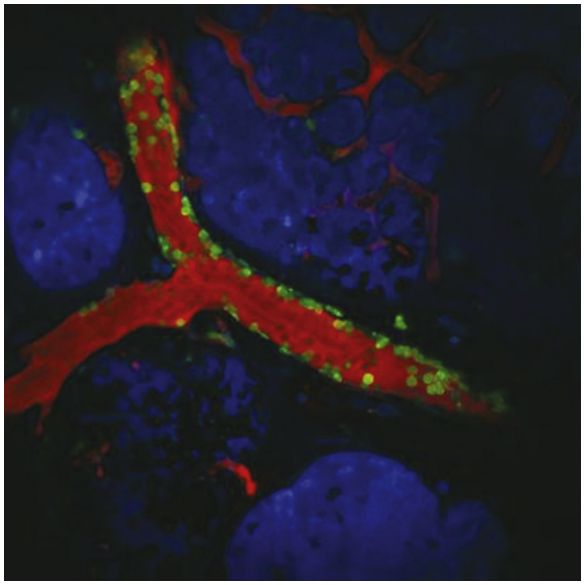


Figure 19-3 Live images of the tumor microenvironment of metastasis (TMEM) in a mouse breast carcinoma TMEM, the direct interaction of a macrophage, migratory tumor cell, and vascular endothelial cell, are sites of intravasation of tumor cells into the circulation. TMEM density correlates with increased risk of distant metastasis in breast cancer patients.[29,30] TMEM are detected in this live image, from a mouse model of breast cancer, as pairs of macrophages *(green)* and tumor cells *(blue)* attached to blood vessels *(red)*, as visualized with a custom-built high-resolution multiphoton microscope. *(Image courtesy of Drs. Allison Harney and John Condeelis. Microscope details obtained from Entenberg D, et al. Setup and use of a two-laser multiphoton microscope for multichannel intravital fluorescence imaging.* Nat Protoc. *2011;6:1500-1520).*

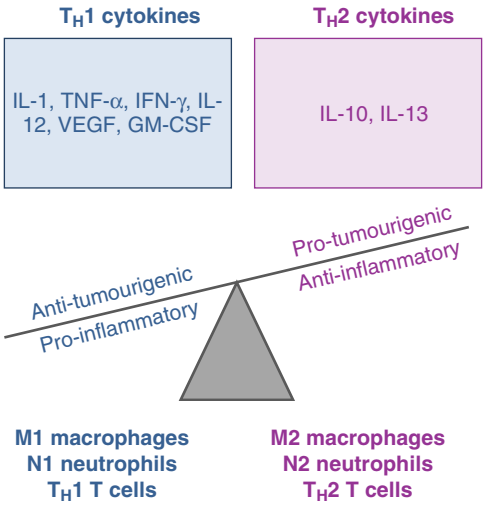


Figure 19-4 The balance of pro- and anti-inflammatory cytokines and cellular states govern cancer outcome Tumor and stromal cells produce a variety of cytokines and chemokines that either contribute to or disrupt the development of a pro-tumorigenic niche. These factors can also reprogram infiltrating immune cells to adopt a pro- or anti-inflammatory state. Generally, M1, N1, and T_H1 cell types produce and respond to T_H1 cytokines and exhibit anti-tumorigenic phenotypes, whereas M2, N2, and T_H2 cell types produce and respond to T_H2 cytokines and exhibit pro-tumorigenic phenotypes. It is likely that this inherent plasticity is important for these cells to reestablish normal homeostasis during inflammation; however, their ability to adapt to different types of microenvironments is hijacked in the tumor niche. *(Image adapted from Coussens LM, Werb Z. Inflammation and cancer.* Nature. *2002;420:860-867).*

live animals.[27] These studies have shown that macrophages are primarily localized in perivascular areas, where they help tumor cells intravasate into the circulation (Figure 19-3).[27-30]

One explanation for the divergent functions of macrophages during normal tissue homeostasis versus tumorigenesis lies in their polarization state. Macrophages are phenotypically plastic. They can alter their polarization status to rapidly accommodate for the needs of different physiological contexts. At the extremes of their phenotypic continuum, macrophages range from M1 to M2 polarization states.[31] "Classically activated" (M1-polarized) macrophages produce type I pro-inflammatory cytokines and participate in antigen presentation, and they play an anti-tumorigenic role in cancer.[2,32] On the other hand, "alternatively activated" (M2-polarized) macrophages produce type II cytokines and anti-inflammatory responses, and they play a pro-tumorigenic role in cancer (Figure 19-4).[2,32] Of note, M2-polarized TAMs have been shown to promote tumorigenesis by providing a major source of proteases and chemokines that support tumor invasion and therapeutic resistance in multiple cancer types.[33-36] For example, it has been shown that TAM-derived cathepsin proteases B and S promote breast cancer growth and metastasis by blunting chemotherapy-induced apoptosis.[34] Furthermore, tumor-secreted cytokines, such as interleukin-4 (IL-4), hijack macrophages in the tumor niche, activating them toward a pro-tumorigenic state.[33,37] Additional characterization of bidirectional interactions between tumor cells and TAMs will likely provide valuable information about how to manipulate the tumor niche in a therapeutic context.

TIE-2–Expressing Monocytes

TEMs are monocytes that express TIE-2, which is a tyrosine kinase receptor for the angiogenic growth factor Angiopoietin. In healthy individuals, TEMs are present at very low levels in the bloodstream and are rarely detected in normal tissue. In contrast, in cancer patients, TEMs are present in higher numbers in the bloodstream and infiltrate neoplastic tissue.[38] TEMs have been implicated in various aspects of tumorigenesis, but they are best known for their role in promoting tumor angiogenesis.[38,39] TEMs regulate angiogenesis by participating in a paracrine signaling loop with Angiopoietin-expressing endothelial cells. This signaling loop is thought to contribute to the inefficacy of anti-angiogenic therapies, such as VEGF-targeted agents. Studies have shown that interfering with the TIE-2/Angiopoietin signaling axis in spontaneous breast or pancreatic

neuroendocrine tumor models significantly inhibits tumor vascularization and blocks tumor growth.[40] These findings suggest that interfering with the TIE-2/Angiopoietin axis may have clinical benefits in reducing tumor vascularization; however, this has not yet been explored in humans.

Neutrophils

Neutrophil granulocytes are the most abundant circulating leukocyte population in humans and play an early role during inflammation by rapidly defending against microorganisms at the site of an infection. Likewise, neutrophils are recruited to tumor sites in response to cytokines and chemoattractants, and the neutrophil:lymphocyte ratio is used as a prognostic indicator of survival and therapeutic outcome in a variety of cancer types.[41-45] At the tumor site, neutrophils have quite diverse roles. For example, it has been shown that neutrophils stimulated with granulocyte colony-stimulating factor (G-CSF) enhance the capacity of circulating tumor cells to seed and grow in secondary sites such as lung.[46] Infiltrating neutrophils were implicated in driving the initial angiogenic switch in pancreatic islet cancers through their ability to activate matrix metalloproteinase (MMP)-9.[47] In contrast, in a recent report on breast cancer by Benezra and colleagues, primary tumor-entrained neutrophils were shown to colonize the premetastatic lung, where they prevented metastatic colonization of disseminated tumor cells via H_2O_2-mediated cell death.[48] In another study, in renal cell carcinoma, it was demonstrated that tumor-secreted chemokines induce recruitment of neutrophils to the premetastatic lung, where they establish a barrier that blunts metastatic colonization.[49] Interestingly, microarray expression analysis revealed that poorly metastatic tumor cells expressed higher levels of these neutrophil chemokines compared to highly metastatic tumor cells, providing a possible explanation for their metastatic inefficiency.[49]

In light of these divergent findings, the role of neutrophils during tumor progression remains unclear. However, similar to the different polarized states of macrophages, it has been shown that neutrophils can acquire N1 or N2 phenotypes, which have very different effects on cancer[50] (see Figure 19-4). The N1 phenotype is pro-inflammatory and elicits an anti-tumoral response by recruiting $CD8^+$ T cells and secreting pro-inflammatory T_H1 cytokines such as tumor necrosis factor-α (TNFα) or IL-12 (see Figure 19-4). In contrast, the N2 phenotype promotes tumorigenesis and plays a role in immune suppression mediated by transforming growth factor (TGF)-β. Indeed, studies have shown that blocking TGFβ signaling can revert pro-tumorigenic N2 neutrophils to an anti-tumorigenic N1 phenotype.[51] This inherent plasticity may explain why neutrophils have been reported to have both pro- and anti-tumorigenic effects in animal models and in patients.[50]

Interestingly, a common side effect of patients undergoing chemotherapy is neutropenia, which is characterized by a significant deficiency in the population of neutrophil white blood cells. This deficiency underlies the extreme risk of infection in cancer patients, as the body loses a major rapid defensive mechanism against invading microorganisms. Recombinant G-CSF protein is frequently used in combination with chemotherapy in cancer patients in order to stimulate the bone marrow to produce more neutrophils.[52] Despite the fact that neutropenia is dangerous for patients because of increased risk of infection, retrospective analyses have reported that neutropenia in response to chemotherapy is correlated with improved overall survival, suggesting that a depletion of systemic neutrophils may be important for optimizing the anti-tumoral effects of therapy.[52,53]

Mast Cells

Mast cells are also derived from myeloid progenitors and act as cellular barriers of infection. Mast cells contain histamine and are best known for their roles during allergic responses and autoimmune diseases. As mediators of inflammation, mast cells become activated in response to tissue injury and release their granules containing histamine, proteases, heparin, prostaglandins, and various cytokines into the microenvironment, where they induce proliferation of nearby endothelial cells. Of note, as tumors hijack inflammatory responses to tissue injury, it is not surprising that mast cells have also been implicated during tumor angiogenesis.[54,55] In patients, studies have shown that mast cells are abundant in the tumor microenvironment and that the degree of mast cell infiltration correlates with microvascular density and poor prognosis.[54,55] Moreover, in animal models of squamous-cell carcinoma and pancreatic islet cancer, oncogene activation was shown to rapidly recruit mast cells, which were essential for induction of angiogenesis via activation of different proteolytic cascades.[56,57]

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are immunosuppressive precursors of dendritic cells, macrophages, and granulocytes and play a role in maintaining normal tissue homeostasis in response to various systemic insults. MDSCs are elevated in the circulation in response to bacterial and parasitic infection[58-60] and are also elevated in the blood of tumor-bearing hosts. Mobilization of MDSCs into the bloodstream is mediated by cytokines that are secreted by tumor cells. For example, studies in breast cancer have shown that TGFβ signaling in tumor cells increases secretion of tumor-derived chemokine CXCL5, which acts as a chemoattractant for CXCR2-expressing MDSCs.[61] Interestingly, the accumulation of MDSCs is a common response to cancer therapy and is thought to contribute to lack of

therapeutic efficacy. For instance, MDSCs have been shown to accumulate in the blood of breast cancer patients receiving doxorubicin-cyclophosphamide chemotherapy and are present in highest numbers in stage IV patients with metastatic disease.[62]

Once MDSCs arrive at the tumor site, their main function is to disrupt major mechanisms of tumor immune surveillance, including antigen presentation and cell-mediated anti-tumor immunity.[21] They achieve immune suppression in multiple ways: First, they have been reported to directly inhibit proliferation and activation of $CD4^+$ T cells and $CD8^+$ T cells.[63,64] Several studies have demonstrated that the immunosuppressive effects of MDSCs on T cells are largely mediated by the production of nitric oxide, among other factors.[64-66] They are also associated with promoting M2-polarization of TAMs to yield a pro-tumorigenic phenotype, as described earlier.[67] MDSCs have also been shown to participate in a paracrine loop with TAMs, whereby MDSC-derived IL-10 (a type II cytokine) causes inhibition of macrophage-derived IL-12 (a type I cytokine), which feeds back to amplify the production of IL-10 from MDSCs.[68] Last, they are capable of impairing NK cell function. Specifically, MDSCs inhibit cytotoxicity of NK cells by reducing production of NK-derived interferon γ (IFNγ) in a TGF-β–dependent manner.[69,70] In light of these findings, it is not surprising that increased peripheral MDSC levels correlate with advanced disease.[62]

Lymphoid Lineage

NK Cells

Natural killer (NK) cells are cytotoxic lymphocytes that play an important role in both innate and adaptive immune responses. NK cells are unique compared to other lymphoid cell types in that they are capable of distinguishing between healthy cells and stressed cells (such as tumor cells) in the absence of antigen presentation.[71] Therefore, it is generally recognized that NK cells provide an anti-tumorigenic immune response. Indeed, reduced NK cell levels in patients with cancer have been shown to correlate with decreased overall survival.[72-74] One way that NK cells can recognize and kill cancer is through "missing-self" activation, whereby MHC class I molecules, which characterize healthy cells and inhibit NK cytotoxicity, are lost from tumor cells. Alternatively, NK cells can recognize cancer through "stress-induced activation," whereby stress-induced ligands are upregulated on tumor cells to activate receptors on adjacent NK cells.[71]

Despite the many anti-tumor roles that NK cells have, it has been recently shown that following surgical removal of tumors in cancer patients, NK cell cytotoxicity is significantly reduced, largely attributed to surgical tissue stress.[75] This effect is correlated with increased metastatic incidence in mouse models, demonstrating that the anti-tumorigenic functions of NK cells can be disrupted with standard-of-care treatment regimens. Therefore, therapies that opt to enhance NK populations following surgery may provide an opportunity for improving therapeutic efficacy at early stages of treatment.

$CD4^+$ Helper T Cells

$CD4^+$ helper T (T_H) cells play diverse roles in cancer. Like polarized macrophages, they can be subdivided into phenotypically divergent T_H1 and T_H2 lineages, directed by secretion of IL-12 and IL-4, respectively.[76] Specifically, T_H1 cells secrete pro-inflammatory cytokines, including IFN-γ, TNFα, IL-2, and IL-12 (see Figure 19-4) to induce antigen presentation on MHC molecules by antigen-presenting cells (APCs), and $CD8^+$ T cell cytotoxicity. They also exhibit direct cytotoxic functions by releasing granules that directly kill tumor cells in their microenvironment.[77] On the other hand, T_H2 cells play a role in humoral immunity. They secrete high levels of anti-inflammatory cytokines, such as IL4-6, IL-10, and IL-13 (see Figure 19-4), and elicit pro-tumorigenic effects through inhibition of $CD8^+$ T-cell cytotoxicity and immunosuppression to promote tumor growth.[77]

Despite the finding that T_H2 cells are generally associated with pro-tumorigenic functions, they may retain some inherent plasticity that allows them to provide beneficial anti-tumor effects. For instance, it has been shown that T_H2 cell infiltrates are positively correlated with disease-free survival in patients with Hodgkin's lymphoma.[78] In other types of cancers, the T_H cell balance seems to be more important for disease outcome. For example, in breast cancer, the ratio of T_H1 to T_H2 cells within the tumor microenvironment correlates with tumor stage and grade, suggesting that the balance of T_H cell types, rather than the degree of infiltration of any one T_H subset, may be useful for predicting prognosis in patients.[79]

Regulatory T Cells (T_{REGS})

Regulatory T cells, or T_{REG} cells, are a unique subtype of $CD4^+FoxP3^+$ T cells that play divergent roles during tumorigenesis. On the one hand, increased T_{REG} cell infiltrate has been correlated with reduced overall survival in various cancers such as breast cancer and hepatocellular carcinoma.[80,81] In these pro-tumorigenic roles, T_{REG} cells function by releasing immunosuppressive factors, such as TGF-β and T_H2 cytokines, which disrupt antigen presentation by APCs and impair the anti-tumorigenic effects of $CD8^+$ cytotoxic T cells and NK cells.[82,83] Furthermore, T_{REG} cells have been shown to impair M1-polarization of macrophages and support immunosuppressive myeloid cells.[84]

On the other hand, it has been shown that infiltration of T_{REG} cells is correlated with improved overall survival in cancers as diverse as colorectal cancer, bladder cancer, and head and neck cancer.[85-87] The mechanisms of these divergent roles remain elusive in the literature; it is not clear whether T_{REG} cells exhibit context-dependent functionality or whether they encompass multiple subpopulations of cell types, with distinct functions, that are not differentiated using conventional markers.[76]

$CD8^+$ Cytotoxic T Cells

Cytotoxic $CD8^+$ T (T_C) cells are lymphocytes that kill cells infected with viruses and also have the capacity to kill tumor cells. They express T-cell receptors (TCRs) on their surfaces, which recognize antigens presented on MHC molecules on APCs. In cancer, infiltration of T_C cells is associated with an anti-tumorigenic capacity across a wide range of cancer types, including melanoma, breast cancer, ovarian cancer, gliomas, and hepatocellular carcinoma, and is associated with prolonged overall survival in patients.[76,88,89] On activation, T_C cells release granules containing cytotoxic factors, for example, perforin and granzyme proteases, which induce apoptosis of infected cells.[83] It has been reported that T_C cells are abundant at the invasive edge of tumors, where they improve patient response to chemotherapy and prognosis.[88]

B Cells

B cells play an important role in humoral immunity and mediate both antibody production and activation of T cells through antigen presentation. They are most abundant at the tumor invasive edge and in adjacent tertiary lymphoid structures.[76] Although infiltration of B cells in tumors tends to be correlated with better disease outcome and prolonged survival in patients, it remains largely unclear whether B cells have positive or negative roles in disease progression based on confounding literature.[90] For instance, it has been reported that B cells elicit pro-tumorigenic effects by secreting tumorigenic cytokines, altering the ratio of T_H1:T_H2 cell infiltrates within tumors, and mediating immune cell recruitment.[91] In addition, studies in breast cancer have shown that B cells influence phenotypic transitions between T-cell states and mediate the conversion of $CD4^+$ T_H cells into T_{REG} cells to promote metastatic dissemination.[92] In contrast, other studies have demonstrated anti-tumorigenic effects of tumor-infiltrating B cells. For example, B cells can release cytotoxic factors to directly kill tumor cells, such as TNFα and the protease granzyme B, and can induce immune suppression through secretion of IL-10.[93] Taken together, these studies suggest that further classification of B-cell subpopulations may explain their diverse functions and may suggest novel therapeutic avenues for disease intervention.

Nonhematopoietic Stromal Cell Types in the Tumor Microenvironment

In addition to the inflammatory cells that constitute the primary tumor and metastatic microenvironment, additional accessory cells in the tumor stroma, such as cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs), engage in a dynamic interplay between immune cells and tumor cells. In the following section, we discuss the role of CAFs and MSCs during tumor progression and how immune cells modulate their function.

Cancer-Associated Fibroblasts

Fibroblasts are a predominant cell type in connective tissue and are responsible for depositing ECM and basement membrane components, regulating differentiation events in associated epithelial cells, modulating immune responses, and mediating wound healing.[94,95] In the tumor microenvironment, fibroblasts are present in aberrantly high numbers and are generally regarded as genomically stable compared to tumor cells.[96] However, CAFs are quite different from normal fibroblasts; it has been reported that normal prostate epithelial cells resemble intraepithelial neoplasia in mice when co-injected with CAFs, but not when co-injected with normal fibroblasts.[97] Once CAFs accumulate in the tumor microenvironment, they are activated by growth factors and cytokines such as TGF-β, platelet-derived growth factor (PDGF), and various proteases.[95,98,99] Following activation, CAFs provide a major source of oncoproteins and growth factors that fuel the growing tumor and modulate immune function.[100,101] For example, CAFs are a significant source of hepatocyte growth factor (HGF) in the tumor microenvironment, which supports angiogenesis, acts as a chemotactic agent for monocytes, and interferes with normal function and maturation of B cells.[102-104]

Mesenchymal Stem Cells

Bone marrow–derived MSCs play an important role during tissue remodeling and repair. They are mobilized into the circulation in response to cytokines released by tissue injury and have a multipotent capacity to give rise to osteoblasts, adipocytes, and chondrocytes.[105] Given that tumorigenesis shares many similarities with the process of wound healing, tumors are likewise able to mobilize and recruit bone marrow–derived MSCs to support remodeling events.[106] Indeed, it has been shown that mobilization of MSCs into circulation is pronounced in patients with advanced breast

cancer and is associated with metastasis and chemoresistance.[107,108] Similar to the divergent roles that immune cells play in cancer, MSCs likewise have seemingly distinct functions. For example, it has been shown that glioma cells co-injected with MSCs into BALB/c-nu/nu mice exhibit a marked reduction of tumor vascularization compared to glioma cells injected with normal astrocytes.[109] In contrast, other studies have demonstrated a positive regulatory relationship between MSCs and tumor angiogenesis, whereby co-injection of MSCs and colon cancer cells into BALB/c-nu/nu mice induces a significant increase in tumor volume and microvascular density.[110] The differential effects of MSCs may be due to their capacity to modulate immune cells and thereby change the landscape and secretome of the tumor microenvironment.[105] Indeed, MSCs have been shown to alter both innate and adaptive arms of the immune system. For example, tumor-associated MSCs have been shown to suppress the pro-inflammatory function of dendritic cells, by blunting their ability to produce TNFα.[101] Furthermore, MSCs can interfere with NK activation and cytotoxicity.[111] Finally, MSCs have been shown to negatively regulate $CD8^+$ T_C cell–mediated cytotoxicity and have a capacity to switch pro-inflammatory T_H1 cells to adopt an anti-inflammatory phenotype.[101,112] Taken together, in addition to the complex relationship between tumor cells and immune cells, additional accessory cells in the tumor-associated stroma contribute to disease progression by influencing tumor-immune interactions.

Summary

Despite the classical role of the immune system in defending against infection and systemic insult, many studies have shown that immune cell functions in the tumor niche often correlate with disease progression. This correlation is largely attributed to the reprogramming effects of the tumor microenvironment, which contains a plethora of growth factors and cytokines that hijack immune cells to play oncogenic roles. Although many immune cells are receptive to tumor-secreted factors and can adopt pro-tumorigenic functions, these cells have also been reported to retain many defensive functions in the tumor microenvironment, speaking to the complexity of tumor–stroma interactions during disease. It is likely that the diverse outcomes of immune cell function are highly context dependent, involving a reprogramming decision that is based on a large variety of factors, including tissue type, tumor secretome, tumor landscape, oxygen availability, tissue pH, and ECM architecture.

The therapeutic implications of tumor microenvironment research are vast. For example, disrupting key tumor–stroma interactions that play known roles during disease progression may interfere with the tumor's ability to exploit immune cells in the microenvironment. Furthermore, attempts have been made to deplete specific immune-cell populations in the tumor environment; however, this type of an approach has been reported to lead to compensatory infiltration of alternative cell types that completely alter the tumor landscape in a way that is unpredictable and often unfavorable.[113,114] Of note, rather than depleting immune cell populations in tumors, it may be advantageous to develop therapies that opt to "re-educate" immune cells in the microenvironment, in order to take advantage of the valuable defense functions of these cell types. For instance, inducing anti-tumorigenic activation states in plastic immune-cell types, such as forcing classical activation of TAMs, may be a unique way to manipulate the tumor microenvironment in a way that re-establishes normal mechanisms of tissue homeostasis. Many of these therapies have been studied or are currently being explored in the laboratory; it will be interesting to observe the evolution of these novel approaches in patients in the future, with the ultimate goal of maximizing positive responses to therapy.

Key References

2. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009;9:239-252.
5. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454:428-435.
10. Bruix J, Llovet JM. Hepatitis B virus and hepatocellular carcinoma. *J Hepatol*. 2003;39(suppl 1):S59-S63.
11. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol*. 2012;13:607-6215.
12. Proctor LM. The Human Microbiome Project in 2011 and beyond. *Cell Host Microbe*. 2011;10:287-291.
13. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet*. 2012;13:260-270.
14. Howitt MR, Garrett WS. A complex microworld in the gut: gut microbiota and cardiovascular disease connectivity. *Nat Med*. 2012;18:1188-1189.
15. Schwabe RF, Wang TC. Cancer. Bacteria deliver a genotoxic hit. *Science*. 2012;338:52-53.
16. Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol*. 2012;9:599-608.
17. Lampe JW. The Human Microbiome Project: getting to the guts of the matter in cancer epidemiology. *Cancer Epidemiol Biomarkers Prev*. 2008;17:2523-2524.
18. DeNardo DG, Brennan DJ, Rexhepaj E, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1:54-67.

19. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res*. 2007;9:212.
20. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11:123-134.
21. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol*. 2009;182:4499-4506.
23. Conejo-Garcia JR, Benencia F, Courreges MC, et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med*. 2004;10:950-958.
25. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 2004;4:71-78.
26. Gocheva V, Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle*. 2007;6:60-64.
27. Wyckoff JB, Wang Y, Lin EY, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res*. 2007;67:2649-2656.
28. Entenberg D, Wyckoff J, Gligorijevic B, et al. Setup and use of a two-laser multiphoton microscope for multichannel intravital fluorescence imaging. *Nat Protoc*. 2011;6:1500-1520.
29. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. 2006;124:263-266.
30. Robinson BD, Sica GL, Liu YF, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res*. 2009;15:2433-2441.
31. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8:958-969.
32. Mantovani A, Sica A, Sozzani S, et al. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25:677-686.
33. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
34. Shree T, Olson OC, Elie BT, et al. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev*. 2011;25:2465-2479.
35. Bell-McGuinn KM, Garfall AL, Bogyo M, et al. Inhibition of cysteine cathepsin protease activity enhances chemotherapy regimens by decreasing tumor growth and invasiveness in a mouse model of multistage cancer. *Cancer Res*. 2007;67:7378-7385.
36. Joyce JA, Baruch A, Chehade K, et al. Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. *Cancer Cell*. 2004;5:443-453.
37. Wang HW, Joyce JA. Alternative activation of tumor-associated macrophages by IL-4: priming for protumoral functions. *Cell Cycle*. 2010;9:4824-4835.
38. Venneri MA, De Palma M, Ponzoni M, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood*. 2007;109:5276-5285.
39. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. 2005;8:211-226.
40. Mazzieri R, Pucci F, Moi D, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell*. 2011;19:512-526.
41. Walsh SR, Cook EJ, Goulder F, et al. Neutrophil-lymphocyte ratio as a prognostic factor in colorectal cancer. *J Surg Oncol*. 2005;91:181-184.
42. Cho H, Hur HW, Kim SW, et al. Pre-treatment neutrophil to lymphocyte ratio is elevated in epithelial ovarian cancer and predicts survival after treatment. *Cancer Immunol Immunother*. 2009;58:15-23.
43. Sarraf KM, Belcher E, Raevsky E, et al. Neutrophil/lymphocyte ratio and its association with survival after complete resection in non-small cell lung cancer. *J Thorac Cardiovasc Surg*. 2009;137:425-428.
44. Shimada H, Takiguchi N, Kainuma O, et al. High preoperative neutrophil-lymphocyte ratio predicts poor survival in patients with gastric cancer. *Gastric Cancer*. 2010;13:170-176.
45. Chua W, Charles KA, Baracos VE, et al. Neutrophil/lymphocyte ratio predicts chemotherapy outcomes in patients with advanced colorectal cancer. *Br J Cancer*. 2011;104:1288-1295.
46. Kowanetz M, Wu X, Lee J, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A*. 2010;107:21248-21255.
47. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci U S A*. 2006;103:12493-12498.
48. Granot Z, Henke E, Comen EA, et al. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell*. 2011;20:300-314.
49. Lopez-Lago MA, Posner S, Thodima VJ, et al. Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression. *Oncogene*. 2013;32:1752-1760.
50. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis*. 2012;33:949-955.
51. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. 2009;16:183-194.
52. Epstein RJ. The CXCL12-CXCR4 chemotactic pathway as a target of adjuvant breast cancer therapies. *Nat Rev Cancer*. 2004;4:901-909.
54. Lin EY, Pollard JW. Role of infiltrated leucocytes in tumour growth and spread. *Br J Cancer*. 2004;90:2053-2058.
56. Soucek L, Lawlor ER, Soto D, et al. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med*. 2007;13:1211-1218.
58. Haile LA, von Wasielewski R, Gamrekelashvili J, et al. Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. *Gastroenterology*. 2008;135:871-881 881 e1–e5.
59. Delano MJ, Scumpia PO, Weinstein JS, et al. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med*. 2007;204:1463-1474.
60. Cuervo H, Guerrero NA, Carbajosa S, et al. Myeloid-derived suppressor cells infiltrate the heart in acute *Trypanosoma cruzi* infection. *J Immunol*. 2011;187:2656-2665.
61. Yang L, Huang J, Ren X, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 2008;13:23-35.
62. Diaz-Montero CM, Salem ML, Nishimura MI, et al. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother*. 2009;58:49-59.
66. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, et al. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer Res*. 2011;71:5101-5110.
67. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol*. 2005;174:636-645.
68. Sinha P, Clements VK, Bunt SK, et al. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol*. 2007;179:977-983.
69. Liu C, Yu S, Kappes J, et al. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood*. 2007;109:4336-4342.
70. Li H, Han Y, Guo Q, et al. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol*. 2009;182:240-249.
71. Vivier E, Ugolini S, Blaise D, et al. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol*. 2012;12:239-252.
72. Qiu H, Xiao-Jun W, Zhi-Wei Z, et al. The prognostic significance of peripheral T-lymphocyte subsets and natural killer cells in patients with colorectal cancer. *Hepatogastroenterology*. 2009;56:1310-1315.
75. Tai LH, de Souza CT, Bélanger S, et al. Preventing post-operative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. *Cancer Res*. 2013;73:97-107.
76 Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12:298-306.
77. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*. 2010;29:309-316.

78. Schreck S, Friebel D, Buettner M, et al. Prognostic impact of tumour-infiltrating Th2 and regulatory T cells in classical Hodgkin lymphoma. *Hematol Oncol*. 2009;27:31-39.
80. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24:5373-5380.
81. Fu J, Xu D, Liu Z, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology*. 2007;132:2328-2339.
82. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol*. 2009;10:689-695.
83. Yang ZZ, Novak AJ, Ziesmer SC, et al. Attenuation of CD8(+) T-cell function by CD4(+)CD25(+) regulatory T cells in B-cell non-Hodgkin's lymphoma. *Cancer Res*. 2006;66:10145-10152.
84. Tiemessen MM, Jagger AL, Evans HG, et al. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci U S A*. 2007;104:19446-19451.
85. Badoual C, Hans S, Rodriguez J, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res*. 2006;12:465-472.
86. Winerdal ME, Marits P, Winerdal M, et al. FOXP3 and survival in urinary bladder cancer. *BJU Int*. 2011;108:1672-1678.
87. Frey DM, Droeser RA, Viehl CT, et al. High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer*. 2010;126:2635-2643.
88. Halama N, Michel S, Kloor M, et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res*. 2011;71:5670-5677.
89. Mahmoud SM, Paish EC, Powe DG, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol*. 2011;29:1949-1955.
90. Erdag G, Schaefer JT, Smolkin ME, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res*. 2012;72:1070-1080.
92. Olkhanud PB, Damdinsuren B, Bodogai M, et al. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. *Cancer Res*. 2011;71:3505-3515.
93. Klinker MW, Lundy SK. Multiple mechanisms of immune suppression by B lymphocytes. *Mol Med*. 2012;18:123-137.
95. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6:392-401.
96. Hosein AN, Wu M, Arcand SL, et al. Breast carcinoma-associated fibroblasts rarely contain p53 mutations or chromosomal aberrations. *Cancer Res*. 2010;70:5770-5777.
99. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298-307.
101. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105:1815-1822.
104. Tyan SW, Kuo WH, Huang CK, et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. *PLoS One*. 2011;6:e15313.
105. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol*. 2008;8:726-736.
106. El-Haibi CP, Bell GW, Zhang J, et al. Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy. *Proc Natl Acad Sci U S A*. 2012;109:17460-17465.
107. Roodhart JM, Daenen LG, Stigter EC, et al. Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. *Cancer Cell*. 2011;20:370-383.
108. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
109. Ho IA, Toh HC, Ng WH, et al. Human bone marrow-derived mesenchymal stem cells suppress human glioma growth through inhibition of angiogenesis. *Stem Cells*. 2013;31:146-155.
110. Zhu W, Xu W, Jiang R, et al. Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. *Exp Mol Pathol*. 2006;80:267-274.
111. Spaggiari GM, Capobianco A, Becchetti S, et al. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood*. 2006;107:1484-1490.
112. Rasmusson I, Ringdén O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation*. 2003;76:1208-1213.
113. Pahler JC, Tazzyman S, Erez N, et al. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia*. 2008;10:329-340.
114. Pyonteck SM, Gadea BB, Wang HW, et al. Deficiency of the macrophage growth factor CSF-1 disrupts pancreatic neuroendocrine tumor development. *Oncogene*. 2012;31:1459-1467.

This page intentionally left blank

References

1. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860-867.
2. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer*. 2009;9:239-252.
3. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet*. 2000;1:57-64.
4. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539-545.
5. Medzhitov R. Origin and physiological roles of inflammation. *Nature*. 2008;454:428-435.
6. Murphy JB, Rous P. The behavior of chicken sarcoma implanted in the developing embryo. *J Exp Med*. 1912;15:119-132.
7. Rous P. A transmissible avian neoplasm. (Sarcoma of the common fowl.). *J Exp Med*. 1910;12:696-705.
8. Martins-Green M, Boudreau N, Bissell MJ. Inflammation is responsible for the development of wound-induced tumors in chickens infected with Rous sarcoma virus. *Cancer Res*. 1994;54:4334-4341.
9. Dolberg DS, Hollingsworth R, Hertle M, Bissell MJ. Wounding and its role in RSV-mediated tumor formation. *Science*. 1985;230:676-678.
10. Bruix J, Llovet JM. Hepatitis B virus and hepatocellular carcinoma. *J Hepatol*. 2003;39(suppl 1):S59-S63.
11. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol*. 2012;13:607-6215.
12. Proctor LM. The Human Microbiome Project in 2011 and beyond. *Cell Host Microbe*. 2011;10:287-291.
13. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet*. 2012;13:260-270.
14. Howitt MR, Garrett WS. A complex microworld in the gut: gut microbiota and cardiovascular disease connectivity. *Nat Med*. 2012;18:1188-1189.
15. Schwabe RF, Wang TC. Cancer. Bacteria deliver a genotoxic hit. *Science*. 2012;338:52-53.
16. Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol*. 2012;9:599-608.
17. Lampe JW. The Human Microbiome Project: getting to the guts of the matter in cancer epidemiology. *Cancer Epidemiol Biomarkers Prev*. 2008;17:2523-2524.
18. DeNardo DG, Brennan DJ, Rexhepaj E, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1:54-67.
19. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res*. 2007;9:212.
20. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11:123-134.
21. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol*. 2009;182:4499-4506.
22. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med*. 2001;7:1194-1201.
23. Conejo-Garcia JR, Benencia F, Courreges MC, et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med*. 2004;10:950-958.
24. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol*. 2002;196:254-265.
25. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 2004;4:71-78.
26. Gocheva V, Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle*. 2007;6:60-64.
27. Wyckoff JB, Wang Y, Lin EY, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res*. 2007;67:2649-2656.
28. Entenberg D, Wyckoff J, Gligorijevic B, et al. Setup and use of a two-laser multiphoton microscope for multichannel intravital fluorescence imaging. *Nat Protoc*. 2011;6:1500-1520.
29. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. 2006;124:263-266.
30. Robinson BD, Sica GL, Liu YF, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res*. 2009;15:2433-2441.
31. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8:958-969.
32. Mantovani A, Sica A, Sozz ani S, et al. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25:677-686.
33. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24:241-255.
34. Shree T, Olson OC, Elie BT, et al. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev*. 2011;25:2465-2479.
35. Bell-McGuinn KM, Garfall AL, Bogyo M, et al. Inhibition of cysteine cathepsin protease activity enhances chemotherapy regimens by decreasing tumor growth and invasiveness in a mouse model of multistage cancer. *Cancer Res*. 2007;67:7378-7385.
36. Joyce JA, Baruch A, Chehade K, et al. Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. *Cancer Cell*. 2004;5:443-453.
37. Wang HW, Joyce JA. Alternative activation of tumor-associated macrophages by IL-4: priming for protumoral functions. *Cell Cycle*. 2010;9:4824-4835.
38. Venneri MA, De Palma M, Ponzoni M, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood*. 2007;109:5276-5285.
39. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. 2005;8:211-226.
40. Mazzieri R, Pucci F, Moi D, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. *Cancer Cell*. 2011;19:512-526.
41. Walsh SR, Cook EJ, Goulder F, et al. Neutrophil-lymphocyte ratio as a prognostic factor in colorectal cancer. *J Surg Oncol*. 2005;91:181-184.
42. Cho H, Hur HW, Kim SW, et al. Pre-treatment neutrophil to lymphocyte ratio is elevated in epithelial ovarian cancer and predicts survival after treatment. *Cancer Immunol Immunother*. 2009;58:15-23.
43. Sarraf KM, Belcher E, Raevsky E, et al. Neutrophil/lymphocyte ratio and its association with survival after complete resection in non-small cell lung cancer. *J Thorac Cardiovasc Surg*. 2009;137:425-428.
44. Shimada H, Takiguchi N, Kainuma O, et al. High preoperative neutrophil-lymphocyte ratio predicts poor survival in patients with gastric cancer. *Gastric Cancer*. 2010;13:170-176.
45. Chua W, Charles KA, Baracos VE, et al. Neutrophil/lymphocyte ratio predicts chemotherapy outcomes in patients with advanced colorectal cancer. *Br J Cancer*. 2011;104:1288-1295.
46. Kowanetz M, Wu X, Lee J, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. *Proc Natl Acad Sci U S A*. 2010;107:21248-21255.
47. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci U S A*. 2006;103:12493-12498.
48. Granot Z, Henke E, Comen EA, et al. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell*. 2011;20:300-314.
49. Lopez-Lago MA, Posner S, Thodima VJ, et al. Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression. *Oncogene*. 2013;32:1752-1760.
50. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: friend or foe? *Carcinogenesis*. 2012;33:949-955.
51. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell*. 2009;16:183-194.
52. Epstein RJ. The CXCL12-CXCR4 chemotactic pathway as a target of adjuvant breast cancer therapies. *Nat Rev Cancer*. 2004;4:901-909.

53. Mayers C, Panzarella T, Tannock IF. Analysis of the prognostic effects of inclusion in a clinical trial and of myelosuppression on survival after adjuvant chemotherapy for breast carcinoma. *Cancer*. 2001;91:2246-2257.
54. Lin EY, Pollard JW. Role of infiltrated leucocytes in tumour growth and spread. *Br J Cancer*. 2004;90:2053-2058.
55. Ribatti D, Vacca A, Nico B, et al. The role of mast cells in tumour angiogenesis. *Br J Haematol*. 2001;115:514-521.
56. Soucek L, Lawlor ER, Soto D, et al. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med*. 2007;13:1211-1218.
57. Coussens LM, Raymond WW, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev*. 1999;13:1382-1397.
58. Haile LA, von Wasielewski R, Gamrekelashvili J, et al. Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. *Gastroenterology*. 2008;135:871-881 881 e1–e5.
59. Delano MJ, Scumpia PO, Weinstein JS, et al. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med*. 2007;204:1463-1474.
60. Cuervo H, Guerrero NA, Carbajosa S, et al. Myeloid-derived suppressor cells infiltrate the heart in acute *Trypanosoma cruzi* infection. *J Immunol*. 2011;187:2656-2665.
61. Yang L, Huang J, Ren X, et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell*. 2008;13:23-35.
62. Diaz-Montero CM, Salem ML, Nishimura MI, et al. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother*. 2009;58:49-59.
63. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J Immunol*. 2001;166:5398-5406.
64. Mazzoni A, Bronte V, Visintin A, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol*. 2002;168:689-695.
65. Young MR, Wright MA, Matthews JP, et al. Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor-beta and nitric oxide. *J Immunol*. 1996;156:1916-1922.
66. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, et al. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer Res*. 2011;71:5101-5110.
67. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol*. 2005;174:636-645.
68. Sinha P, Clements VK, Bunt SK, et al. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol*. 2007;179:977-983.
69. Liu C, Yu S, Kappes J, et al. Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. *Blood*. 2007;109:4336-4342.
70. Li H, Han Y, Guo Q, et al. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol*. 2009;182:240-249.
71. Vivier E, Ugolini S, Blaise D, et al. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol*. 2012;12:239-252.
72. Qiu H, Xiao-Jun W, Zhi-Wei Z, et al. The prognostic significance of peripheral T-lymphocyte subsets and natural killer cells in patients with colorectal cancer. *Hepatogastroenterology*. 2009;56:1310-1315.
73. Takeuchi H, Maehara Y, Tokunaga E, et al. Prognostic significance of natural killer cell activity in patients with gastric carcinoma: a multivariate analysis. *Am J Gastroenterol*. 2001;96:574-578.
74. Villegas FR, Coca S, Villarrubia VG, et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer*. 2002;35:23-28.
75. Tai LH, de Souza CT, Bélanger S, et al. Preventing post-operative metastatic disease by inhibiting surgery-induced dysfunction in natural killer cells. *Cancer Res*. 2013;73:97-107.
76. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12:298-306.
77. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*. 2010;29:309-316.
78. Schreck S, Friebel D, Buettner M, et al. Prognostic impact of tumour-infiltrating Th2 and regulatory T cells in classical Hodgkin lymphoma. *Hematol Oncol*. 2009;27:31-39.
79. Chin Y, Janseens J, Vandepitte J, et al. Phenotypic analysis of tumor-infiltrating lymphocytes from human breast cancer. *Anticancer Res*. 1992;12:1463-1466.
80. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24:5373-5380.
81. Fu J, Xu D, Liu Z, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology*. 2007;132:2328-2339.
82. Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol*. 2009;10:689-695.
83. Yang ZZ, Novak AJ, Ziesmer SC, et al. Attenuation of CD8(+) T-cell function by CD4(+)CD25(+) regulatory T cells in B-cell non-Hodgkin's lymphoma. *Cancer Res*. 2006;66:10145-10152.
84. Tiemessen MM, Jagger AL, Evans HG, et al. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci U S A*. 2007;104:19446-19451.
85. Badoual C, Hans S, Rodriguez J, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res*. 2006;12:465-472.
86. Winerdal ME, Marits P, Winerdal M, et al. FOXP3 and survival in urinary bladder cancer. *BJU Int*. 2011;108:1672-1678.
87. Frey DM, Droeser RA, Viehl CT, et al. High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients. *Int J Cancer*. 2010;126:2635-2643.
88. Halama N, Michel S, Kloor M, et al. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer Res*. 2011;71:5670-5677.
89. Mahmoud SM, Paish EC, Powe DG, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol*. 2011;29:1949-1955.
90. Erdag G, Schaefer JT, Smolkin ME, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res*. 2012;72:1070-1080.
91. Qin Z, Richter G, Schüler T, et al. B cells inhibit induction of T cell-dependent tumor immunity. *Nat Med*. 1998;4:627-630.
92. Olkhanud PB, Damdinsuren B, Bodogai M, et al. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. *Cancer Res*. 2011;71:3505-3515.
93. Klinker MW, Lundy SK. Multiple mechanisms of immune suppression by B lymphocytes. *Mol Med*. 2012;18:123-137.
94. Tomasek JJ, Gabbiani G, Hinz B, et al. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol*. 2002;3:349-363.
95. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6:392-401.
96. Hosein AN, Wu M, Arcand SL, et al. Breast carcinoma-associated fibroblasts rarely contain p53 mutations or chromosomal aberrations. *Cancer Res*. 2010;70:5770-5777.
97. Olumi AF, Grossfeld GD, Hayward SW, et al. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res*. 1999;59:5002-5011.
98. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res*. 2001;264:169-184.
99. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298-307.
100. Fukumura D, Xavier R, Sugiura T, et al. Tumor induction of VEGF promoter activity in stromal cells. *Cell*. 1998;94:715-725.
101. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105:1815-1822.

102. Galimi F, Cottone E, Vigna E, et al. Hepatocyte growth factor is a regulator of monocyte-macrophage function. *J Immunol*. 2001;166:1241-1247.
103. van der Voort R, Taher TE, Derksen PW, et al. The hepatocyte growth factor/Met pathway in development, tumorigenesis, and B-cell differentiation. *Adv Cancer Res*. 2000;79:39-90.
104. Tyan SW, Kuo WH, Huang CK, et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. *PLoS One*. 2011;6:e15313.
105. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol*. 2008;8:726-736.
106. El-Haibi CP, Bell GW, Zhang J, et al. Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy. *Proc Natl Acad Sci U S A*. 2012;109:17460-17465.
107. Roodhart JM, Daenen LG, Stigter EC, et al. Mesenchymal stem cells induce resistance to chemotherapy through the release of platinum-induced fatty acids. *Cancer Cell*. 2011;20:370-383.
108. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
109. Ho IA, Toh HC, Ng WH, et al. Human bone marrow-derived mesenchymal stem cells suppress human glioma growth through inhibition of angiogenesis. *Stem Cells*. 2013;31:146-155.
110. Zhu W, Xu W, Jiang R, et al. Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. *Exp Mol Pathol*. 2006;80:267-274.
111. Spaggiari GM, Capobianco A, Becchetti S, et al. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood*. 2006;107:1484-1490.
112. Rasmusson I, Ringdén O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation*. 2003;76:1208-1213.
113. Pahler JC, Tazzyman S, Erez N, et al. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia*. 2008;10:329-340.
114. Pyonteck SM, Gadea BB, Wang HW, et al. Deficiency of the macrophage growth factor CSF-1 disrupts pancreatic neuroendocrine tumor development. *Oncogene*. 2012;31:1459-1467.

Andrea Califano

20

Cancer Systems Biology: The Future

Over the past decade, complementary and at times antithetic views of tumor initiation and progression have emerged, often based on the introduction of novel high-throughput technologies for the characterization of the cell's genetic and epigenetic landscape. On the one hand, the availability of a comprehensive map of the human genome has allowed the development of gene expression profiling techniques, mostly microarray based, to monitor the dynamic state of RNA transcripts in cancer cells. These efforts have revealed the existence of molecularly distinct subtypes of morphologically indistinguishable tumors, often associated with differential outcome,[1] progression,[2] and chemosensitivity.[3] They have also helped identify key genetic programs that are consistently activated (e.g., proliferation, migration, immunoevasion), inactivated (apoptosis, senescence), or frequently modulated (adhesion, angiogenesis, etc.) in tumorigenesis.[4,5] On the other hand, genome-wide studies of both heritable and somatic human variability have moved from theoretical concept to practical reality, opening a new window on both the heritable and the somatic components of cancer etiology. Yet, even as we achieve increased sensitivity in the identification of recurrent somatic alterations for several of the major tumor types, elucidation of the mechanistic role of genetic variability in cancer remains, overall, an elusive target.

Despite these advances, the relationship between genetic alterations and activation/inactivation of specific genetic programs contributing to cancer subtypes remains poorly understood, and the precise cascade of molecular events leading to tumorigenesis and progression is largely uncharted. For instance, although the mesenchymal subtype of glioblastoma is now universally accepted as a distinct subtype, only relatively rare mutations in the NF1 gene appear to co-segregate with it, and the mechanism by which NF1 drives the subtype has not been elucidated[6] (Figure 20-1). Similarly, despite massive sequencing efforts, many mutations discovered in diffuse large B-cell lymphoma fail to precisely co-segregate with its two main functional subtypes, the activated B-cell (ABC) and germinal center B-cell (GCB) phenotypes, which are associated with differential outcome.[7] Even in very common tumors, such as prostate cancer, the repertoire of genomic alterations that contribute to the indolent versus the more aggressive tumors is still unknown.[8] Critically, because of impractical requirements for cohort sizes[9] and lack of methodologies that maximize power for such detection, few epistatic interactions and low-penetrance variants have been identified so far.[10]

This chapter introduces a set of novel approaches and strategies, mostly developed over the past decade, for the elucidation of mechanisms associated with cancer initiation, progression, and chemosensitivity that, overall, go under the name of cancer systems biology. A fundamental departure from the previous methodologies is that, instead of being driven by the isolated analysis of a specific data modality, such as genomic alterations or gene expression profiles, the new discipline is both highly integrative and, more importantly, model driven. By the latter term, we mean that cancer-related datasets are analyzed using small- or large-scale models of the cellular machinery that is most likely to have generated it. These models are still in their infancy and are largely imperfect and incomplete. Yet, even in this embryonic state, they are starting to provide significant new insight and dissecting power, which is only going to increase as the models become more accurate and comprehensive.

Specifically, a key challenge for previous methods, such as genome-wide association studies (GWAS), is lack of statistical power once datasets become truly genome wide. Indeed, given the very large number of somatic events routinely discovered in cancer genomes, including mutations, translocations, gene fusions, aberrant copy number changes, and structural rearrangements,[11] distinguishing "drivers" from "passengers" is challenging and often impossible on a purely statistical basis (Figure 20-2). Not surprisingly, the tumors where greater progress has been made are those with somewhat more benign mutational landscapes, such as leukemias and lymphomas. Still, a significant fraction of these tumors lacks an appropriate causal genetic characterization or mechanistic elucidation of the relationship between genetic alterations and molecular phenotypes. The same can

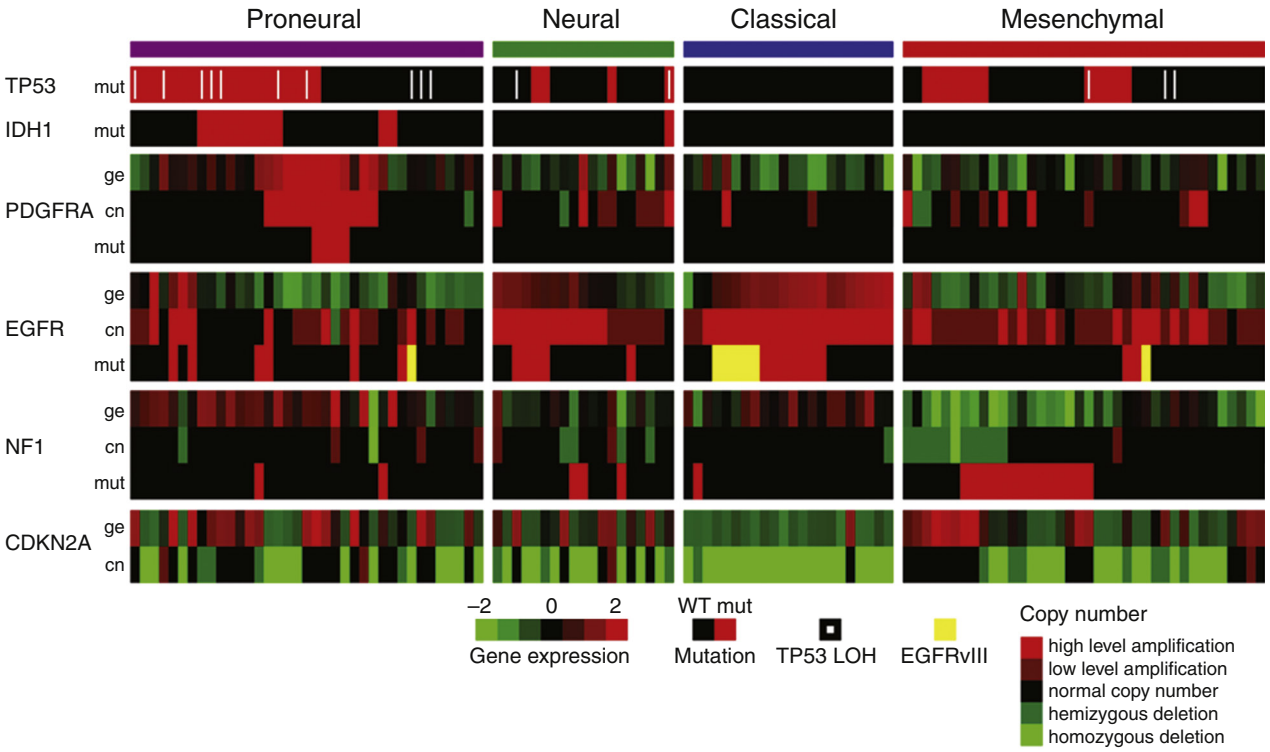


Figure 20-1 Genomic alterations in glioma co-segregate with only some of the identified molecular subtypes. *(With permission from Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1.* Cancer Cell. *2010;17:98-110).*

be said for other genetic or epigenetic data modalities, from gene expression to DNA methylation profiles, which produce long lists of candidate genes with no intrinsic prioritization.

Recently, alternative approaches to those pursued using GWAS statistical approaches have started to emerge.[12] The rationale for these methods is that genome-wide regulatory models representing causal molecular interactions in the cell—for example, transcription factors regulating their transcriptional targets or protein kinases activating their substrates—may help us identify a relatively small number of candidate genes, upstream of genetic programs that are dysregulated, which may be tested for genetic and epigenetic alterations (Figure 20-3).

Variants of such a genetic genomics approach were pioneered in plants[13] and metabolic disease[14] and have been used successfully in cancer-related studies. For instance, identification of the novel HUWE1-MYCN-DLL3 cascade in brain tumors was possible by reverse engineering post-translational modulators of MYCN activity as well as its downstream targets using reverse engineering algorithms.[15] Similarly, the role of RUNX1 as a tumor suppressor mutated in T-cell acute lymphoblastic leukemia (T-ALL) was elucidated based on its most significant overlap with the TLX1 and TLX3 oncogene regulatory programs.[16] In some cases, a network-based view of cancer biology may allow elucidation of the dependency of a phenotype on an entire collection of genetic events, which would be virtually impossible to dissect using statistical approaches. For example, it was recently shown that deletion of any combination of 13 genetic loci distributed across the entire genome leads to functional inactivation of PTEN in glioma patients, via a novel interaction mechanism involving competitive endogenous RNA (ceRNA).[17] Indeed, cancer systems biology applications have exploded over the past 3 years, with studies ranging from the study of key drivers of tumorigenesis in melanoma[18,19] to the dissection of tyrosine kinase signals downstream of ERBB receptors.[20,21]

Such a regulatory-model–driven view of cancer biology is thus emerging as an important *systems-level* contribution to the study of this disease. By taking a more holistic view of tumor-related processes, anchored in gene regulatory mechanisms, cancer systems biology mediates the genetic and the genomic views of cancer to provide novel insight into its mechanisms. Specifically, the proponents of these approaches argue that among all genetic and epigenetic alterations in a tumor, those contributing to its initiation, progression, or drug sensitivity cannot affect regulatory interactions in a random way but must co-segregate within specific regulatory subnetworks that are thus globally dysregulated across different samples of a given tumor subtype. Hence, if the full complement of regulatory interactions regulating the behavior of a specific cancer cell population were known, then it

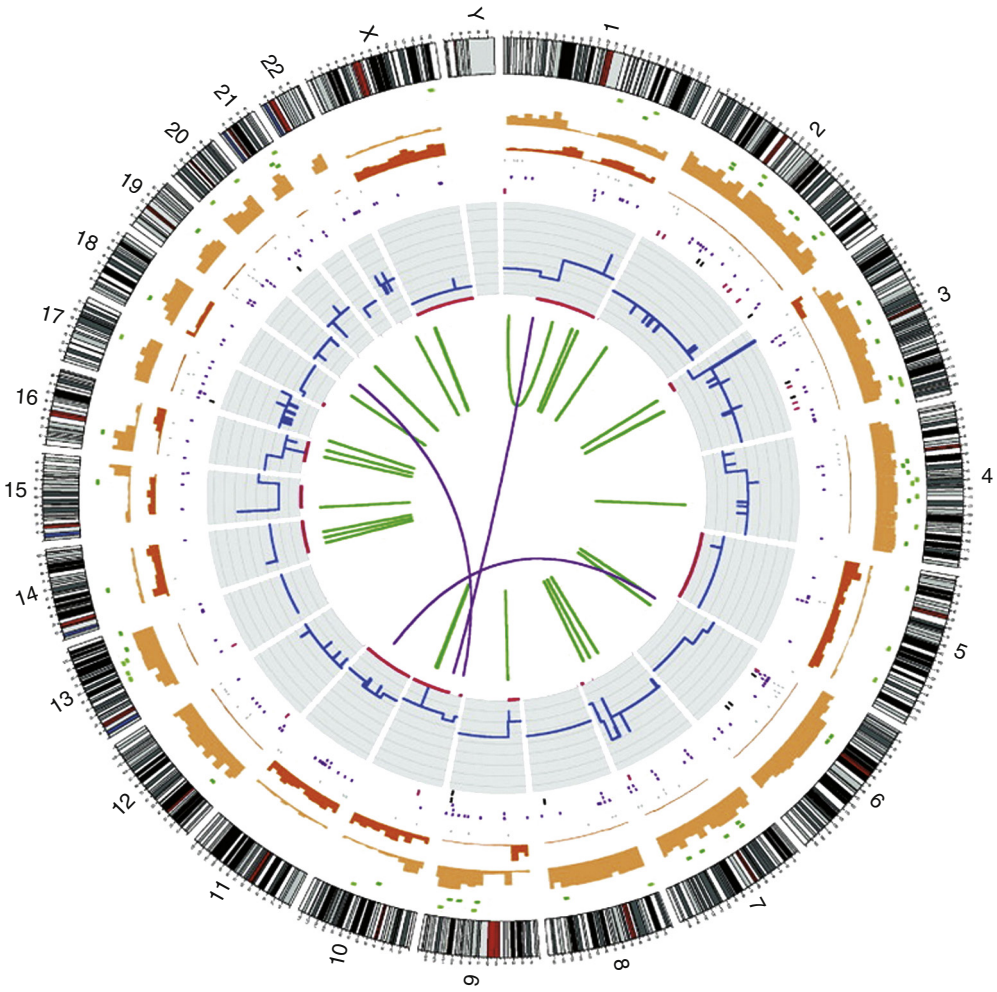


Figure 20-2 Circos plot showing the whole-genome catalogue of somatic mutations from the malignant melanoma cell line COLO-829 This genome has approximately 30,000 somatic base substitutions and 1000 somatic insertions and/or deletions. In coding exons, 272 somatic substitutions are present, including 155 missense changes, 16 nonsense changes, and 101 silent changes. The numbers and types of mutations are highly variable across different cancer genomes. Chromosome number and karyotype are indicated on the exterior of the plot. Key: *blue lines*, copy number across each chromosome; *red lines*, sites of loss of heterozygosity (LOH); *green lines*, intrachromosomal rearrangements; *purple lines*, interchromosomal rearrangements; *red spots*, nonsense mutations; *green spots*, missense mutations; *black spots*, silent mutations; *brown spots*, intronic and intergenic mutations (merged). *(With permission from Garnett M, McDermott U. Exploiting genetic complexity in cancer to improve therapeutic strategies.* Drug Discov Today. *2012;17:188-193).*

should be able to use its structure to separate driver from passenger alterations. The example of RUNX1 in T-ALL[16] is particularly revealing in this case. Here, the functional role of RUNX1 mutations could only be elucidated after determining that its targets are virtually overlapping with those of two previously established oncogenes, TLX1 and TLX3.[22] Without this regulatory insight, it would have been impossible to identify these mutations as statistically significant across the full repertoire of genes.

A key issue, then, is how to assemble accurate and comprehensive repertoires of molecular interactions to create a quantitative regulatory model that may be interrogated to elucidate drivers of tumor-related phenotypes. This is an important question, because virtually all cancer-related publications today contain appealing graphical presentations of molecular pathways in cancer. These bona fide models could provide a starting point for a systems-level study of cancer, as proposed, for instance, by pathway-wide association study (PWAS) strategies.[23]

Unfortunately, knowledge of molecular pathways governing physiological and tumor-related traits is still very poor. Indeed, canonical cancer pathways are more reflective of the researcher's desire to understand biological processes as a relatively linear and interpretable set of events than of the true complexity of cellular regulation. Specifically, these representations have two major limitations. First, they are not context specific. For instance, the EGFR pathway would be identically represented for a glioma and for a lung-cancer cell.

Second, they constitute a manually curated collection of published facts, of which several are actually incorrect, and which represents less than 1% of the total complement of regulatory interactions in the cell. Hence, their use introduces a strong bias toward what is already known (prior knowledge). Indeed, in the absence of a prior hypothesis, interrogation of canonical cancer pathways has been largely unsuccessful in the elucidation of novel tumor-related mechanisms. To understand the difference between a true regulatory network and a canonical cancer pathway, consider Figure 20-4, *A*, showing the differential phosphorylation of canonical EGFR pathway proteins in the H1650 cell line, where EGFR has an activating mutation, compared to the average of all cell

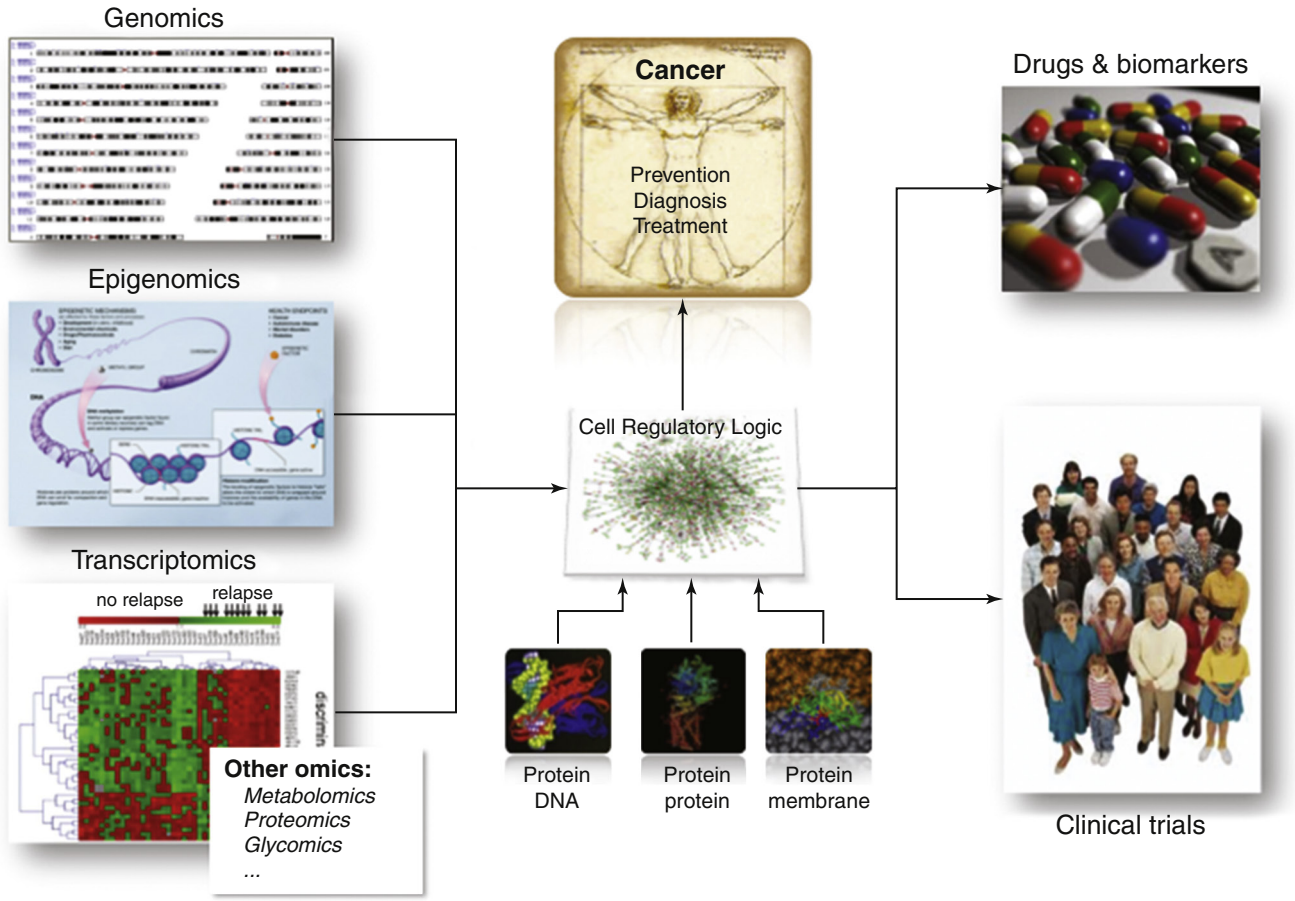


Figure 20-3 The -omics layers of the cell both encode and are processed by a context-specific regulatory logic. At the atomic level, this logic is implemented via molecular interactions, such as protein-DNA, protein-protein, protein-RNA, and RNA-RNA. Dissection and interrogation of this logic in context-specific fashion, using systems biology approaches, is starting to allow elucidation of driver genes responsible for the presentation of relevant cancer-related phenotypes.

lines. In contrast, Figure 20-4, *B*, shows the differentially phosphorylated proteins for the same cell lines in a signal transduction network, inferred de novo from a large-scale collection of phosphopeptide profiles of non–small-cell lung adenocarcinoma.[24] Whereas the pathway-based representation provides no clue that the EGFR pathway may be dysregulated, the network-based representation shows a clear hyperphosphorylated protein pattern surrounding both EGFR and MET.

In the following, we discuss the idea of a simultaneous, de novo reconstruction of context-specific gene regulatory networks from large-scale molecular profile data, and of the genetic and epigenetic variability they harbor and mediate. A classic systems biology workflow generally involves three steps: First is acquisition of molecular profiles for a variety of molecular species, several of which represent gene products, from mRNA to phosphopeptide abundance, as well as of genetic and epigenetic alterations. Second is data integration and reconstruction of the regulatory models for the specific cellular context of interest. The final step is regulatory model interrogation, using genetic and genomic signatures that represent the cellular states of interest. Given the abundance and prior coverage of molecular profile data for cancer, we concentrate on the two latter steps.

Reverse Engineering Regulatory Networks

From a systems biology perspective, cell behavior is driven by the processing of endogenous and exogenous signals and maintenance of homeostasis by a complex network of molecular interaction, that is, the regulatory model of the cell. The latter consists of several cross-interacting layers, including transcriptional, posttranscriptional, signal transduction, stable protein-complex formation, and metabolic interactions. Disruption of network topology or dynamics, within one of these layers or, more frequently, across layers, can aberrantly reprogram the cell by activating specific genetic programs, with the potential outcome of a stable phenotypic transformation such as is observed in tumorigenesis. Systems

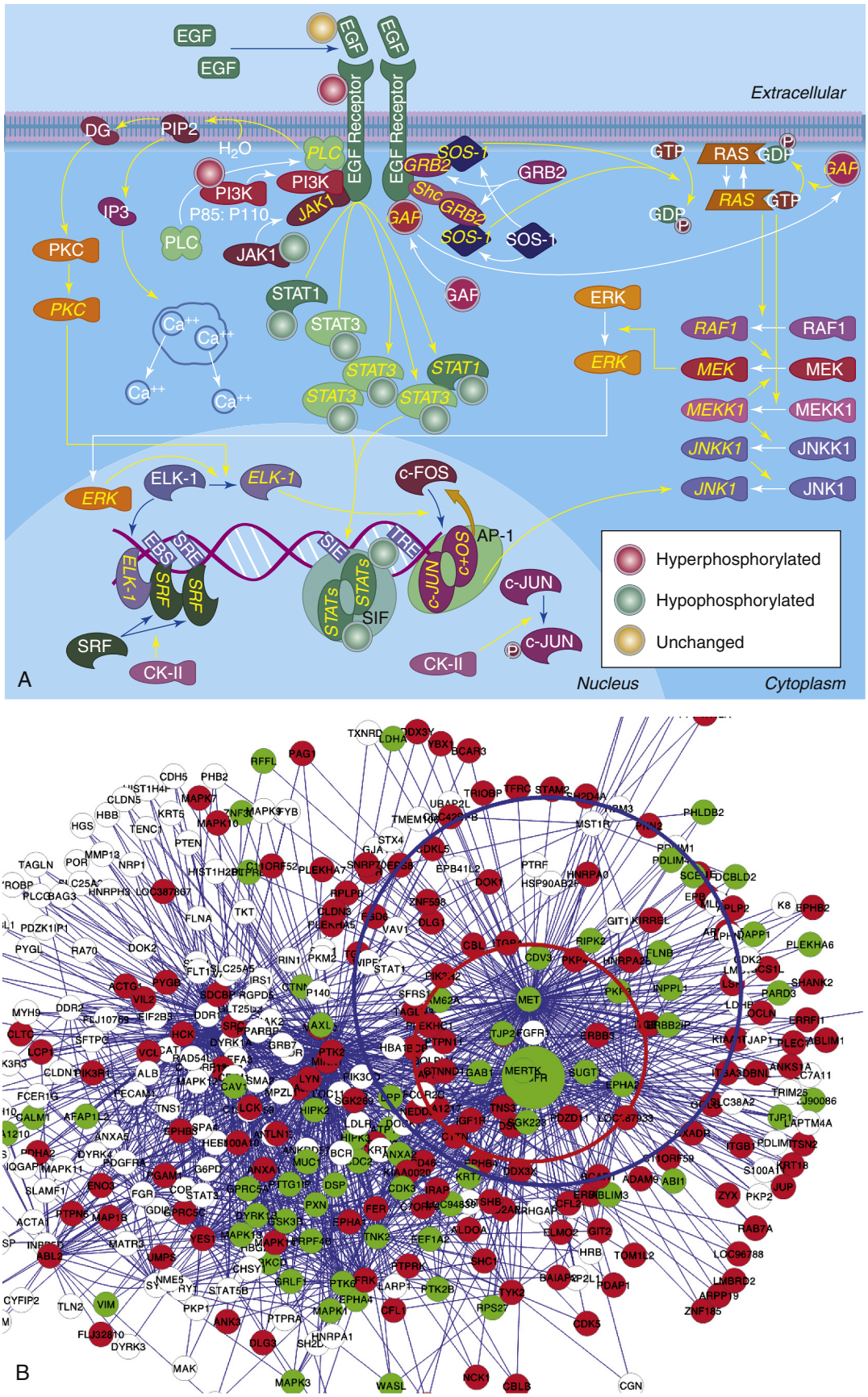


Figure 20-4 Pathway-based vs. network-based representation of differential protein phosphorylation in H1650 cells. (A) Pathway-based representation of differential EGFR pathway protein phosphorylation in H1650 cells, harboring an EGFR-activating mutation. Proteins tagged with a *red circle* are hyperphosphorylated, those tagged with a *green circle* are hypophosphorylated, and those with an *orange circle* are unchanged. Phosphopeptide abundance for the remaining proteins was not detected. **(B)** Network-based representation of differential protein phosphorylation in H1650 cells. Signal transduction network was inferred de novo from a large phospho-proteomic dataset[24] for non–small-cell adenocarcinoma. *Red* proteins are hyperphosphorylated, whereas those in *green* are hypophosphorylated. The *red* and *blue circles* represent EGFR and MET substrates, respectively.

biology, as a field, has evolved on the premise that these regulatory models, from simple kinetic models describing a handful of genes to probabilistic models of genome-wide regulation, could be dissected or "reverse engineered" from experimental data to infer their topology and behavior. One should be aware, however, that regulatory interactions in the cell are both dynamic and context dependent.[25] For instance, the Stat3 transcription factor must be phosphorylated to be transcriptionally active. Hence, the presence or absence of kinase activity or upstream signals may activate or inactivate its role as a transcriptional regulator in dynamic fashion. Of course, one could generate a fully representative, multivariate model of regulation that would represent both states of the transcription factor, but this requires the ability to detect changes in the pairwise interactions between the transcription factor and its targets, as the result of the presence or absence of other molecular species.[26] In addition, the complete model is likely so complex and unyielding that it may be more convenient to use simpler, contextualized models of regulation.

Over the past decade, multiple strategies have been developed by systems biologists to reconstruct the regulatory networks of living cells. Initially, these efforts have been driven by the study of yeast and bacteria as simple model organisms.[27-29] One advantage in these organisms is that regulatory regions on the genome, that is, regions where transcription factors and other chromatin binding proteins bind and regulate gene expression, are relatively short, allowing the efficient use of sequence information in reverse engineering. For instance, in yeast, promoter regions have an average length of 600 bp, whereas human genes may have distal regulatory elements hundreds of kilobases away from the transcription start site. In addition, gene regulation in higher eukaryotes is made dauntingly more complex by the presence of alternative splice variants, alternative start sites, and multiple poly-A tails.

Fortunately, as data generation technology and computational algorithms advance, regulatory models are becoming increasingly quantitative and predictive, thus capturing regulation of biological process more precisely. Currently, reverse-engineering methods can be mostly grouped into four categories. The following is not intended to provide a comprehensive description of all reverse engineering approaches in systems biology, but rather to provide a more general understanding of key differences between approaches.

Optimization-Driven Machine Learning Approaches

Because of the high-dimensional nature of the regulatory space covered by molecular profiles and the comparatively small number of distinct molecular profiles available in tumor repositories, such as those assembled by the Cancer Genome Atlas (TCGA) consortium[30] and the Catalogue of Somatic Mutations in Cancer (COSMIC),[31] classical methods such as maximum likelihood are not directly applicable to inferring causal relationships between regulators and regulated gene products. However, several assumptions, such as maximum parsimony, have allowed the successful use of machine learning (ML) approaches.[32] In this context, ML addresses this problem by asking what is the regulatory model with the largest posterior probability to have generated the observed molecular profile data. This cannot be addressed by enumerating all the possible models, of course. As a result, many approaches rely on greedy algorithms and underlying approximations, such as assuming that regulatory models can be effectively represented as directed acyclic graphs (DAG) that lack feedback loops.[27] The final model can then be used to infer systems behavior inferences with future data.[32] Examples of such methods include the analysis of regulators of gene expression modules,[33] as well as the use of Bayesian and dynamic Bayesian networks for reverse engineering transcriptional[27,34] and signal-transduction networks.[35] For a general review of these methods, see Refs. 36 through 38. Factors that affect the precision of the predictions by ML approaches include the dataset quality, feature preselection for single residues, and algorithm selection based on the purpose and data type.[39]

Integration of Prior Knowledge and Experimental Evidence

Rather than predicting interaction from a single data modality, such as gene expression profile data, systems biologists have embraced the vast number of repositories containing experimental data from high-throughput approaches. These range from gene expression profiles, to genome-wide chromatin immunoprecipitation data (GW-ChIP), to yeast-two-hybrids and nuclear pull-down assays. Although partial and often inaccurate in isolation, the knowledge contained in these repositories can be effectively integrated into a single unified model, using computational models to combine the probability about a specific event (e.g., the interaction between two molecular species) from a wealth of independent facts. For instance, transcriptional interactions may combine data from GW-ChIP, DNA binding site motif analysis, and co-expression, among a number of other relevant data types. Use of ML frameworks for the integration of multiple weak clues, from naïve Bayes classifiers,[40] to Bayesian networks,[41] to a variety of consensus scoring methods, has been very successful in generating more accurate and comprehensive molecular interaction models.[42,43] Recently, an intriguing result has been shown in the analysis

of the Dialogue on Reverse Engineering Assessment Methods (DREAM) challenges.[44] DREAM is an attempt to objectively measure the ability of computational approaches to correctly infer facts about regulatory network structure. Specifically, it was shown that integration of the results of many different inference algorithms performs generally better or at least as well as the best individual algorithm. This is an important result, as we often do not have a principled approach to objectively assess the quality of each given method and may instead want to use the integrative results of several of them. An additional value of integrative methods is that they allow the integration of completely heterogeneous types of data. For instance, it was recently shown that protein structure information from x-ray and nuclear magnetic resonance (NMR) crystallography can be effectively integrated with functional data to accurately predict protein-protein interactions.[45] For a more comprehensive review of integrative approaches, see Ref. 46.

Regression Analysis

Regression techniques have long been used to estimate parameters for kinetic models from experimental data and could, at least in theory, be extended to the inference of parameters for entire regulatory models. Various regression methods have been proposed for pathways or network inference, including maximum likelihood,[47,48] least squares,[49-51] and Bayesian inference,[52-54] to obtain estimates of model parameters.[55] Maximum likelihood approaches infer parameter values from a distribution, as those maximizing the posterior probability of the experimental data; least squares approaches determine the parameter values that minimize the sum of the squares of the residuals, that is, the difference between each experimental and model data point; and finally, Bayesian methods use a priori models for the unknown parameter distribution to compute their most likely values. A key problem of regression methods is that they are generally underdetermined. A determined problem is one where the number of independent observations of a system is equal to the number of parameters that must be estimated. Overdetermined problems—that is, those with more observations than parameters—have the advantage that estimates have some level of statistical robustness. When the number of parameters is much larger than the number of observations, however, an infinite number of parameter values becomes equally possible, thus requiring other heuristics. This is a common issue in real biological systems, as the number of parameters for a system with tens of thousands of interacting molecular species can be in the several hundred thousands, but few datasets with more than a few hundred independent experimental profiles are available for the same system. To address this problem, a number of dimensionality reduction approaches have been developed, which work by either splitting a single high-dimensional problem into a number of independent lower-dimensional ones, such as via singular value decomposition, or by penalizing models with a larger number of connections via sparsity constraints.[56]

Information Theory and Probabilistic Methods

Shannon's information theory provides a probabilistic model to characterize information flow in a system.[57] In recent years, information theory has become a staple of systems biology approaches,[58] for instance, by predicting the minimal machinery in the cell necessary to account for the globally observed information transfer between distinct molecular species characterized by a profile, such as mRNA,[26,59,60] mutational data,[61] and microRNAs.[17,62]

In general, these approaches view a regulatory process, such as a signaling pathway or a transcriptional regulatory circuit, as a flow of information carried by a cascade of molecular events, each one adding some noise to the process. Shannon entropies are estimated for each node of the cascade to quantify the information flow, and gain or loss of information is used as evidence to derive the structure of the information circuit. Specifically, the concept of mutual information (MI)[63] or conditional mutual information (CMI)[64] is usually applied to quantify two-way and three-way functional dependencies between variables, respectively. MI measures the mutual dependence of two random variables, without any linearity assumptions, for instance, between the mRNA of a transcription factor and one of its potential targets. CMI measures whether the information transfer between two variables is dependent on a third variable, for instance, the availability of a protein kinase affecting the ability of a transcription factor to regulate its targets. Various probabilistic and information-theoretic methods have been proposed for the reverse engineering of regulatory networks, of which some have been extensively experimentally validated, such as ARACNe,[59,65] MINDy,[26] Hermes,[66] minet,[67] and CLR.[68]

ARACNe and MINDy: A Case Study

These two algorithms are among those with the most extensive repertoire of experimental assays supporting their ability to dissect regulatory networks in cancer cells, as well as among the most adopted by the research community. We thus use them as a case study to illustrate several relevant concepts in the area of reverse engineering. Other examples of extensively validated algorithms with applications to the

study of cancer and other diseases include CONEXIC,[19] CLR,[18] and Bayesian networks[14,35]; see Refs. 12 and 69 for a more detailed review.

ARACNe has been widely applied to dissect transcriptional regulatory networks from gene expression profiles of multiple cancer subtypes, including B-cell lymphoma,[65] breast cancer,[70] glioma,[71] and T-cell leukemia.[16] ARACNe first estimates the statistical significance of the mutual information between every possible transcription factor and candidate target ($TF_A \rightarrow T$) and then removes candidate interactions that violate the data processing inequality, an information-theoretic property stating that information transferred via a direct interaction must exceed that transferred via any indirect path (e.g., $TF_A \rightarrow TF_B \rightarrow T$). Thus, if even one indirect path is found that exceeds the MI computed for a specific transcription factor–target pair, the candidate direct interaction is eliminated. ARACNe was experimentally validated by ChIP and GW-ChIP assays, as well as by silencing of transcription factors followed by gene expression profiling; see the papers referenced earlier.

This analysis was extended to higher-order interactions. Specifically, the Modulator Inference by Network Dynamics (MINDy) algorithm was developed to identify posttranslational modulators of transcription factor activity on their targets using the conditional mutual information.[26,72] These include both co-transcription factors and upstream signaling proteins regulating the transcription factor activity.[26] Genome-wide searches based on this methodology were successful in identifying the HUWE1 ubiquitin ligase as a key modulator of MYCN in neural stem cells,[15] as well as the serine threonine kinase STK38 as a key modulator of MYC in human B cells,[73] among several other interactions that were experimentally validated.

Interrogating Pathways and Networks

Once regulatory models such as pathways and networks are available, machine learning approaches can harness them to study cancer phenotypes. For example, a random forests algorithm was used to perform pathway-based SNP analysis to predict cancer-patient survival outcome.[74] Applied to multiple myeloma, using Affymetrix 500K SNP array data, the method connected informative SNPs and patient survival, thus identifying a candidate pathway involved in modulating patient survival and associated genetic variants.[74] A multitude of other machine learning algorithms have also been used to assist in GWAS analysis, such as SNP discovery,[75,76] SNP interaction,[77,78] cancer subtype classification,[79] survival outcome association,[80] and key genetic variant identification[81,82]; see Ref. 83 for a more comprehensive review. Yet, a common characteristic of these approaches is that the associations they identify are generally statistical in nature and not easy to relate to a specific molecular mechanism. This is mostly the result of using pathways that are universal, sparse generalizations of regulatory processes in human cells, rather than regulatory models that are cell specific.

Several approaches are now emerging that simultaneously address the reconstruction of context-specific cell regulatory models as well as their interrogation to identify drivers of both physiological and pathological phenotypes; see Refs. 12 and 69 for a more complete review. For instance, the Master Regulator Inference algorithm (MARINa; Figure 20-5)

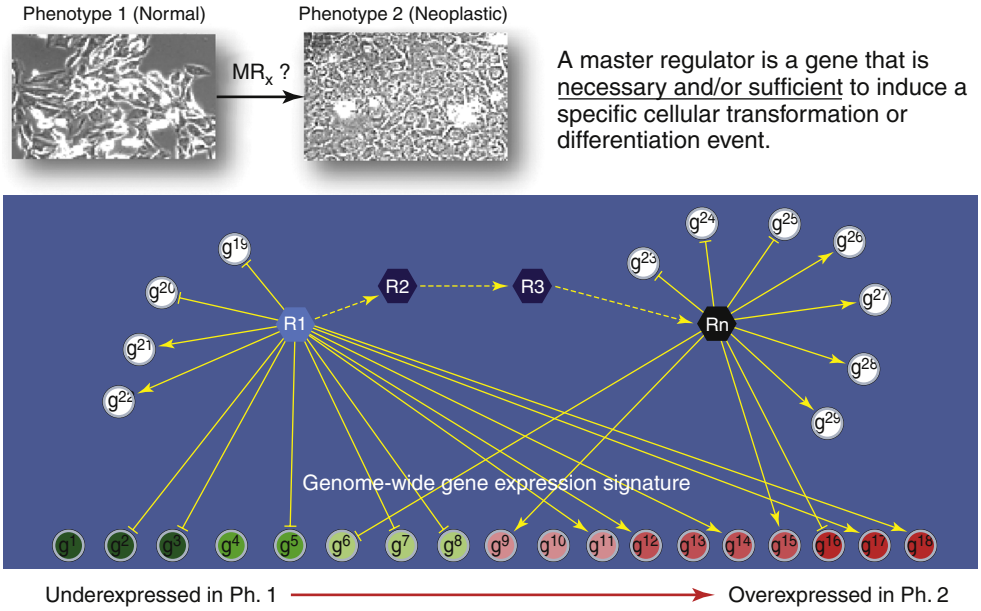


Figure 20-5 Discovery of master regulator genes using the MARINa algorithm Master regulators (e.g., R_1) are genes whose activated and repressed transcriptional targets are highly enriched in genes that are over- and underexpressed in the transition from the first to the second phenotype, respectively. Other regulators (e.g., R_n) have targets that are not enriched in differentially expressed genes.

was used to discover genes and associated mechanisms that are causally related to the implementation of a specific phenotype.[40,70,71] Specifically, rather than asking what genes are differentially expressed in a specific cellular phenotype, MARINa uses a context-specific, causal regulatory model to identify candidate master regulator (MR) genes that regulate the majority of genes that are differentially expressed. Given a small set of candidate MRs, experimental validation can then identify those both necessary and/or sufficient to implement the specific phenotype. This approach was used, for instance, to elucidate CEBPB/CEBPD and STAT3 as synergistic master regulators of the mesenchymal subtype of human glioblastoma, as well as FOXM1 and MYB as MRs of the germinal center reaction.

Similar approaches have led to the identification of NFκB as a key addiction point for the ABC subtype of diffuse B cell lymphoma tumors, associated with worst prognosis.[7,84] Indeed, while NFκB is never mutated in this phenotype, thus lacking the necessary prerequisites to elicit oncogene addiction,[85] it constitutes a natural functional bottleneck by integrating aberrant signals from several genetic alterations in upstream pathways, including the *Card11*, *Tnfaip3*, *Traf2*, *Traf5*, *Map3k7*, and *Trank1* genes, that induce cell addiction to its function. Here, we use the term *non-oncogene addiction* to indicate dependencies on genes that are not genetic drivers (i.e., mutated oncogenes) but are rather induced by upstream genetic alterations and aberrant signals.[86] Similarly, different integrative network-based systems biology approaches were used to identify drivers of tumor initiation[19] and differential patient survival[18] in melanoma. Although these constitute very recent developments, the availability of methods to assemble accurate and cancer-specific regulatory models will determine an explosion in this kind of approach.

Recent data[16,40,70,71] suggest that a majority of cancers present regulatory bottleneck structures, where aberrant signals from genomic alterations are integrated by a relatively small number of genes to implement specific downstream regulatory programs that contribute to define the cancer cell transcriptional identity and to ensure its survival and replicative potential (Figure 20-6). These programs are collectively known as the hallmarks of cancer.[87]

Causal Interactions: Reducing the Search Space for Genetic and Epigenetic Variability

One of the limitations of current associative methods is that the search space is so vast that the statistical correction required to account for testing so many hypotheses can mask all but the most obvious single genetic associations. In complex diseases such as cancer, where multiple genetic events are necessary to induce transformation and progression, this all but prevents testing for complex epistatic interactions. As a result, despite extensive sequencing of cancer genomes, most cancer cases lack a clear heritable or somatic explanation, and the co-factors contributing to tumorigenesis even when key oncogenes are elucidated are mostly unknown. For instance, although we know that MYC translocations, HER2 amplifications, and APC deletions contribute to tumor etiology in Burkitt's lymphoma, breast cancer, and colon cancer, respectively, these lesions are not sufficient to induce tumorigenesis and require a number of co-mutation events to induce transformation.

Systems biology approaches can help in this context by dramatically reducing the search space or by increasing the statistical significance using complementary, statistically independent evidence. This can be accomplished using two strategies. First, identification of functional master regulators of tumor-related phenotypes can dramatically reduce the search space for potential genetic and epigenetic alterations only to genes encoding proteins that interact with or modulate the activity of master regulators. Second, by integrating the probability of a gene having a functional effect on the phenotype, based on regulatory network analysis, with the probability deriving from GWAS-type studies, for instance. using Fisher's or Stouffer's method to integrate *p*-values, one can dramatically increase the statistical significance of

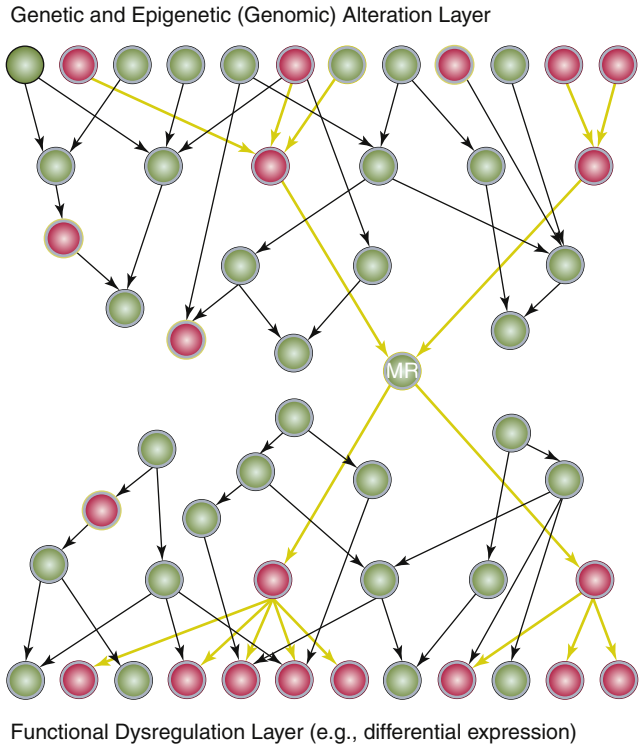


Figure 20-6 Master regulators implement the hall marks of cancer. One or more regulatory bottlenecks integrate aberrant signals from a spectrum of genetic alterations through key regulators (master regulators) that in turn implement the genetic programs necessary for tumor initiation and progression (cancer hallmarks).

relevant genes, thus exceeding the high bar determined by multiple hypothesis testing correction. A number of such methods have already been presented at cancer meetings, and more are likely to be published in the near future.

An illustrative example is the discovery of a new layer of regulation implemented by competitive endogenous RNAs (ceRNA). These are RNAs that compete through high-affinity RNA binding sites for recruitment of the same microRNA. When the abundance of one ceRNA in a pair decreases, the bound microRNAs are freed and can target the other species, thus repressing it. Although this regulatory layer was first hypothesized in plants,[88] its potential impact on human disease was only recently demonstrated when it was shown that the PTEN pseudogene is a tumor suppressor by regulating PTEN as its cognate ceRNA.[89] Moreover, by combined sequence and gene expression analysis,[90] as well as by extending the MINDy algorithm,[17] it was shown that ceRNA constitutes a vast regulatory network capable of dysregulating not only PTEN, but also the majority of oncogenes and tumor suppressors. For PTEN, in particular, this analysis reduced the number of regulators whose genetic deletion could affect PTEN abundance to about 500 loci, thus leading to the identification of 13 loci frequently deleted in TCGA glioma patients, such that co-deletion of any combination of these loci would lead to significant PTEN inactivation by microRNA-mediated repression.[17] These combinations of locus deletions could not possibly have been identified on a statistical basis, given the relatively small number of TCGA patients (approximately 500) in the glioblastoma cohort.

The powerful integrative nature of systems biology approaches requires multidimensional datasets and, more importantly, datasets in which multiple data modalities are profiled for the same samples, such as those assembled by recent, large consortia such as TCGA.[91] When used in integrative fashion with other large datasets containing GWAS and other data, such as the database of Genotypes and Phenotypes (dbGAP)[92] and the Gene Expression Omnibus (GEO), the real value of this new discipline can start to be fully harnessed.

Using Regulatory Networks to Elucidate Drug Activity

The naïve researcher may think of drugs as highly selective agents, affecting specific substrates that are relevant in equally specific disease-related contexts. For instance, imatinib is best known as a potent inhibitor of the BCR-ABL fusion protein in chronic myelogenous leukemia (CML), resulting from the Philadelphia chromosome translocation.[93] Yet, most drugs affect (directly or indirectly) a large repertoire of substrates by both high- and low-affinity interactions. Furthermore, drugs are metabolized by biological systems, resulting in a spectrum of metabolites, some inert and some having differential target affinity and potency. As a result, drug discovery and characterization could gain a tremendous advantage from systems biology approaches, which study a drug's activity and effects in the context of the full regulatory context that mediates compound activity. These approaches have the potential of transforming drug repositioning, identification of potential toxicity, drug sensitivity prediction, and combination therapy from trial-and-error efforts to a quantitative and predictive methodology.

Consider drug repositioning, for instance. Of the repertoire of more than 1500 distinct drugs approved by the U.S. Food and Drug Administration (FDA), it is likely that many could have far-ranging application in cancer, outside of the narrow disease confines they were originally approved for, frequently for non–cancer-related applications. For instance, imatinib, originally approved for CML, is now finding significant reuse as a C-KIT protein kinase inhibitor in gastrointestinal stromal tumors.[94] Indeed, repositioning of existing drugs in alternative disease systems is believed to have great potential to significantly shorten the drug development period and strengthen drug pipelines.[95,96] Similarly, in the context of combination therapy, testing the synergistic potential of just the 1500 FDA-approved drugs would require screening 1,125,000 combinations, a number that is far too vast to be efficiently tested across a broad base of tumor types and diseases.

Although the development of systems-biology–based methodologies for the study of drug-related phenotypes is far less developed than its use for elucidating driver genes, a handful of approaches have been proposed that suggest an important role for this area of research. For instance, experimental perturbations of the SOS pathway were used to elucidate drug mechanisms of action in *Escherichia coli*,[28] and chemogenomic profiles were analyzed to study antibiotic resistance using network-based approaches.[97] Similarly, network-based mechanisms of action of small compounds were elucidated using the Interactome-based Drug Enrichment Analysis (IDEA) algorithm,[98] while a library of gene expression profiles following drug perturbation was instrumental in the study of compounds that could induce a desired gene expression signature in the cell[99] and machine learning approaches were used to elucidate the molecular basis of drug resistance in yeast.[100] These are just some notable examples, as the literature on systems-level analysis of small-molecule activity is increasing exponentially.

In the context of drug repositioning, a Bayesian factor regression model was developed to perform off-target drug

repositioning based on transcriptional responses,[53] and a consensus analysis based on the connectivity map was successful in identifying experimentally validated analogs of existing drugs.[101] More recently, an elegant, small-scale kinetic model of signal transduction was used to elucidate a caspase-8–dependent mechanism inducing time-dependent synergy between erlotinib, an EGFR inhibitor, and doxorubicin in triple-negative breast cancers,[102] suggesting that regulatory models are becoming sufficiently mature to allow time- and dosage-dependent analysis.

Taken together, these early examples are illustrative of the great relevance that regulatory network–based analysis will have in the prediction of drug activity, in the rescue of drug sensitivity, and in the in silico exploration of combination therapy approaches.

Recent Trends and Future Perspectives

As discussed earlier, a variety of research strategies have emerged in cancer systems biology since the discipline's conceptual inception, about a decade ago.[103,104] Although the initial forays in this area were mostly of a theoretical nature, over the past 5 years these approaches have achieved significant success and gained considerable traction, especially in the elucidation of complex, multigene mechanisms that would not have been approachable using conventional molecular biology strategies. In its coming of age, systems biology has become one of the most powerful methods for the high-throughput generation of testable hypothesis, frequently achieving a greater than 50% likelihood of experimental validation, something that would have been previously unthinkable from purely computational approaches. For instance, validation of transcription factor targets, microRNA targets, and protein-protein interactions predicted by reverse engineering methods is frequently in the 80% range and thus competitive with high-throughput experimental approaches.[17,26,45,59] Similarly, a number of disease mechanisms have been recently elucidated, following validation of regulatory network–based predictions.* These are only a few representative examples from a now-vast literature.

In spite of these successes, concerns and skepticism are still pervasive in the biological community on the role and effectiveness of systems-biology approaches in dissecting complex diseases, such as cancer, and in elucidating novel therapeutic strategies for translational research. To address these concerns, this nascent discipline must make progress on two critical aspects. First, novel technologies are critically needed to interrogate cellular systems in integrative fashion; and, more importantly, translation of concepts emerging from systems approaches must lead to clinical applications. Indeed, these two aspects are critically related. For instance, although a genomic view of biology has now been around for more than 30 years, its translational applications have been largely the result of our newfound ability to exploit large-scale genomic information originating from the map of the human genome and from those of other organisms. Given the glacial times required for clinical experimentation, compared to molecular biology research in the lab, the first clinical applications of the genomic revolution are only now starting to appear, a full 10 years after the completion of the human genome map. Systems biology, as a discipline, is much more recent, and it would be unreasonable to expect immediate translation of its findings. Like genomics, this discipline could not achieve its true potential until appropriate datasets and technologies became available. As a result, the first attempts to translate cancer systems-biology discoveries are only now starting to percolate to the clinic. As in the case of genomic sciences, several years may be necessary before a compelling argument for clinical applications of systems biology can be made. For instance, the Cancer Target Discovery and Development (CTD^2) network,[86] a recent initiative to adopt a high-throughput, systems-biology–based approach to the discovery and validation of novel druggable targets for several tumor subtypes, associated biomarkers, and small-molecule modulators, has been very successful, producing dozens of new experimentally validated targets and small molecules that are now being prioritized for clinical research. The identification of a disease target, of associated biomarkers for population stratification, and of candidate small-molecule inhibitors has traditionally taken 10 years. Several CTD^2 projects were successful in achieving this target during the first 2 years of the program, dramatically accelerating the preclinical discovery process but also linking therapeutic strategies and biomarkers to specific biological mechanisms in vivo, thus ultimately increasing the probability of successful clinical translation.

Perhaps the most critical contribution to the ability to adopt a systems approach to accelerate preclinical discovery has derived from the development and introduction of novel technologies that allow us to both perturb and monitor cellular systems both in vitro and in vivo, as well as from single cells to tissues. These have produced both the comprehensive datasets that power systems biology approaches and the high-throughput validation methodologies to take full advantage of systems biology as an equally high-throughput hypothesis-generating science. Indeed, it is precisely the combination of high-throughput and integrative data acquisition,

* 14-16,22,40,69,71,73,105-107.

hypothesis generation, and experimental validation that has contributed most to the success of this discipline.

The Technologies of Cancer Systems Biology

We are currently experiencing dramatic acceleration in the ability to produce critically needed technologies to rapidly and accurately perturb cellular systems, monitor their changes at the molecular level, and experimentally validate molecular hypotheses. These technological improvements range from chemical biology approaches to perturb cellular systems in a controlled environment—including genome-wide libraries for RNAi-mediated gene silencing, ORF-based cDNA libraries for the genome-wide ectopic expression of genes, and small-molecule libraries of bioactive compounds—to the use of imaging for the detection of phenotypic changes in single cells, to the development of novel approaches to produce proteome-wide readout of peptide and phosphopeptide abundance, to the development of inexpensive approaches for monitoring the activity of hundreds of genes in cellular populations. Interestingly, many of these technologies have more than one use. For instance, pooled RNAi screens, where shRNA libraries are used in a cellular population such that each cell receives a single shRNA on average, are being used both to create datasets for the integrative discovery of cancer driver genes and, using smaller, more targeted libraries, to validate candidate driver genes both in vitro and in vivo.

The availability of data from cellular perturbation assays is critical for implementing systems-biology approaches to elucidating novel molecular mechanisms. This is because interactions for molecular species that are constant across multiple observations cannot be dissected using computational methods. On the other hand, when molecular species abundance is variable across samples, molecular interactions introduce constraints, resulting in highly nonrandom patterns in the data. These patterns can be analyzed and used to infer the most probable set of molecular interactions that originated them. Three distinct approaches to perturb molecular systems have been used in systems biology. The first and most effective one is based on the naturally occurring genotypic variability between individuals in a species or related cancer subtypes, further modulated by exogenous events such as temperature, nutrient intake, or chemical stressors. For instance, a set of gene expression profiles from approximately 400 samples of normal and tumor-related human B cells, covering 18 subtypes of lymphoma, were used to reverse engineer the first experimentally validated transcriptional network for a human cell.[59] The second type of variability arises from genetic manipulations aimed at silencing or expressing specific genes. For instance, profiles from yeast knockout libraries were successfully harnessed to reverse engineer yeast-specific regulatory networks; see Ref. 108 for an example. Finally, gene expression profiles following small-molecule perturbations, such as those in the connectivity map,[99] are also being used to reverse engineer human regulatory networks. Notably, regulatory models produced by analysis of naturally occurring variability in samples from primary tumor tissue are best suited to study driver genes, as they reflect regulation under the full context of autocrine, paracrine, endocrine, and contact signals that cancer cells receive. They also best reflect tumor heterogeneity. Conversely, models produced by chemical perturbations in vitro are optimally suited to dissect small-molecule mechanisms of action, effectors, and genes modulators of sensitivity. Indeed, several laboratories are actively attempting to match tumor dependencies elucidated from natural variability datasets with small-molecule activity and mechanisms elucidated from in vitro perturbations.

In the following paragraphs we discuss two of the most important and innovative technologies embraced and frequently developed by systems biologists, as well as their potential for the implementation of a predictive systems-level approach to cancer prevention, diagnosis, and therapy.

RNAi-Mediated Silencing

Traditional cancer research strategies focus on the use of molecular profiles to discover genes causally responsible for cancer initiation, progression, and drug sensitivity phenotypes, generally known as *driver* genes. Unfortunately, most genes identified by these methods are not causal; rather, these genes are just statistically associated with the specific cancer-related phenotype, also described as *passenger* genes. To simultaneously evaluate the relevance of a large number of genes in the context of a specific cancer-related phenotype, thus identifying a handful for further mechanistic elucidation, a variety of RNAi screening approaches have been developed.[109-111] These technologies, including siRNA and shRNA-based approaches, have provided powerful and efficient tools for screening a large number of genes. Importantly, when RNAi screens are integrated with additional experimental measurements, such as transcription factor activity, protein expression, and protein kinase/phosphatase activity, RNAi-based genetic screens can provide important clues about the structure and organization of regulatory networks, such as transcriptional and signaling networks, controlling a specific phenotype.[112]

shRNA-based RNAi screens are generally used for long-term culture assays, such as clonogenic assays and drug resistance assays. An increasingly popular approach is the pooling of multiple shRNA constructs—by using either multiple hairpins for the same genes, or a large hairpin library covering a fraction of or the complete expressed genome—to infect cells in a single assay.[113-115] This can, for instance, help in the identification of genes that confer sensitivity to a specific drug or in genes that constitute critical addiction point for the tumor cell. Large shRNA library screens have been successfully performed to identify tumor suppressors such as NF1 in neuroblastoma,[116] oncogenic MYD88 mutations in human lymphoma,[117] and haploinsufficient cancer gene RPS14 for 5q− syndrome in anemia and acute myelogenous leukemia.[118] Similarly, an siRNA-based synthetic lethality screen identified a repertoire of genes that sensitize lung cancer cells to paclitaxel, an approved cancer drug, but at 1000-fold lower concentration.[119]

RNAi screens are also well suited to high-content imaging analysis, where entire plates with 96 or 384 cell-silencing assays can be analyzed by high-throughput multicolor confocal microscopy to detect more complex phenotypes, such as cytoskeleton remodeling, cell morphology changes, and lineage differentiation.[119-121] For instance, a genome-wide siRNA screen for cell cycle, size, and proliferation, using quantitative fluorescence microscopy, identified more than 1000 cell-cycle–related genes.[122]

Project Achilles constitutes the most comprehensive attempt to use pooled RNAi technology to explore cellular dependencies in cancer.[123] It provides a summary of 102 cell lines perturbed with genome-wide pooled shRNA libraries representing 11,194 genes. Interestingly, when genetic screens are performed on such a diversity of cellular contexts, they achieve a more universal value that transcends that of the individual cell line analyses and provides a novel and highly compelling new paradigm for systems approaches. For instance, using data from Project Achilles and other related efforts, one may consider whether a specific gene dependency is detected repeatedly in the presence of a specific genetic alteration(s) across a large, heterogeneous panel of cell lines. This could be used to identify regulatory bottlenecks and to dissect the structure of transcriptional and signal transduction pathways. Similarly, by integrating such pooled approaches with chemosensitivity profiles across a number of cell lines, novel insight into small-molecule mechanisms of action, synergy, and genes that may modulate sensitivity could be gained.[3] Finally, by intersecting results from computational analysis of cancer driver genes with results from pooled screen assays, one could further identify small sets of candidate genes that would have a much higher probability of eliciting oncogene or non-oncogene addiction in vivo. Thus, the combination of genetic screens and computational approaches into an integrative systems-biology methodology has significant potential to increase the efficiency and specificity of target and biomarker discovery. For example, computationally inferred genetic backgrounds and gene regulatory networks in primary human tumors have been shown to greatly help in designing the genetic screen strategy and efficiently identifying malignant-state driving genes.[115]

Single-Cell Analysis and Tumor-Cell Heterogeneity

Traditional profiling techniques provide snapshots of molecular species' abundance averaged on populations containing thousands to millions of cells, thus capturing only average behavior. Unfortunately, cancer is a highly heterogeneous disease, both in cell type composition—because tumor cells are mixed with stroma, infiltrating leukocytes, and endothelial cells—and in terms of clonality of the tumor cells, with individual clones differing on a broad range of genetic and epigenetic alterations.[124] Intratumor heterogeneity is also due to differences in subclones, microenvironment, cell cycle stage, and stochastic fluctuations.[125] As a result, regulatory networks or gene signatures inferred from cellular populations may not be representative of any of the individual cells but only of their average common properties.[126,127]

Tumor heterogeneity greatly affects disease progression, transfection and infection rates, as well as drug response,[128] as subpopulations will be filtered over time, and even small clonal components presenting the most optimal fitness will grow exponentially over time, thus eventually dominating tumor composition. For instance, rather than presenting an even transduction efficiency across cells, some Jurkat cell clones consistently exhibit partially efficient RNAi-mediated silencing while others show complete inhibition of the target gene.[129] In such cases, the averaged population is a poor representative of the behavior of the individual clonal subpopulations. We thus need to port the tools of molecular biology for perturbing, profiling, and sensing to the single-cell context. Thanks to recent advances, it is now possible to isolate and profile genetic, genomic, proteomic, and metabolomic features in single cells. These technologies include microfluid-based cell culturing and profiling, high-performance cell sorting, super-resolution and high-content microscopy, single-cell next-generation sequencing, and antibody-based microchamber sensing devices, among many others. For example, whole-genome amplification (WGA) based on the multiple displacement amplification (MDA) method was adapted to single-cell exome sequencing to profile cells from JAK2-negative myeloproliferative neoplasm[130] and clear-cell renal-cell carcinoma.[131] These data showed surprising intratumor heterogeneity in the mutation spectra, significantly

more complex than expected, while providing candidate drivers of tumor progression. Importantly, several of these results could not have been identified from bulk analysis of cell populations.

Multiparameter flow cytometry has been commonly used in single-cell analysis for immunology, stem-cell, and cancer research. For example, causal signaling networks were inferred with a Bayesian network machine learning method by analyzing multiparameter flow-cytometry data from a large number of chemically perturbed immune system cells.[35] Similarly, single-cell measurements of phosphoproteins in acute myeloid leukemia cells obtained by multiparameter flow cytometry were used to differentiate tumor-cell populations in the same patient.[132] These approaches were successful in associating different genetic mutational backgrounds with specific drug response profiles and signal transduction patterns. These results are informative of tumor pathology and drug response mechanisms in tumor-cell subgroups.[132]

Fluorescence-based flow cytometry is limited to monitoring up to a dozen parameters in parallel, thus limiting the range of analytes for network reconstruction and interrogation. A novel mass spectrometry–based single-cell cytometry technique, which uses antibodies covalently bonded with a repertoire of rare earth isotopes (CyTOF), can currently measures up to 50 parameters in parallel at a rate of 100,000 cells per 3-minute interval[133] and will likely soon achieve higher multiplexing. CyTOF was successfully applied to profiling key proteins in cell cycle regulation[134] and to immunophenotyping individual tumor cells, including human leukemia cell lines and leukemia patient samples.[133] These accomplishments are widely regarded as the first forays of systems-level approaches into the domain of single-cell regulation and identity.

Other innovative single-cell profiling techniques have also been widely used to obtain genomic, genetic, proteomic, and metabolomic data. These techniques for nucleic acid profiling include microfluid-based "lab-on-a-chip" devices,[135] new high-throughput polymerase chain reaction (PCR) with or without preamplification,[136,137] and DNA/RNA-Seq.[138] The proteomic and metabolomic profiling techniques that were not discussed earlier include mass spectrometry–based methods (reviewed in Ref. 139) and fluorescence imaging coupled with flow cytometry that measures up to 1000 prelabeled targets.[128] Furthermore, a great range of new technologies is continuously emerging, providing ever finer detail to single-cell biology; see review articles.[140]

In summary, single-cell analysis is providing a new, previously inaccessible layer of information that is instrumental in elucidating the stochastic nature of molecular processes, population variability, and tumor heterogeneity using the quantitative tools of systems biology.

Personalized Systems Medicine: A Patient-centric View of Cancer Research

Extensive research efforts in past decades, especially following the introduction of high-throughput-omics profiling technologies, have clearly highlighted tumor heterogeneity as a result of the highly diverse repertoire of genetic and epigenetic alterations that define the disease. Although we have discussed heterogeneity at the level of single cells, an equally critical challenge of cancer research is patient-to-patient molecular heterogeneity, both in the tumor and in the patient background germline landscape, even when tumors are classified as morphologically indistinguishable. Two critical consequences of such heterogeneity are that patients with tumors that would be considered identical may have dramatically different outcome and response to therapy. For instance, the vast majority of prostate cancers never progresses to aggressive metastatic disease. Unfortunately, the small fraction that does can be indistinguishable from the majority that does not, prompting aggressive and often invasive therapy in patients who would not need it.

As a result, the development of personalized medicine approaches has become an almost inevitable trend in cancer research. Initially, the focus has been on identifying molecularly distinct subtypes of a major tumor class and associating them with equally distinct prognostics and therapeutics. For instance, tumor subtype and activation of specific pathways have been shown to be predictive of anticancer treatment in breast cancer and pancreatic ductal adenocarcinoma.[141,142] Unfortunately, individual tumor phenotypes appear increasingly as the intersection of several layers of regulatory control, including cell of origin, genetic and epigenetic alterations, distal endocrine signals, and the balance of distinct paracrine signals originating from the balance of cellular subpopulations that constitute the tumor microenvironment. As a result, with the possible exception of some leukemias, where somatic perturbation of lineage differentiation events plays a dominant role, a clear demarcation between tumor subtypes is becoming increasingly elusive, and much overlap between even relatively well-defined molecular subtypes is becoming evident, as demonstrated, for instance, by recent attempts to restructure established breast-cancer subtypes.[143] Indeed, for some large tumor classes, such as prostate cancer, a clear subtype distinction has been difficult, and only very recent results hypothesize different basal and luminal progenitor origins as a possible discriminating event.[144]

Now that full genomic and epigenomics characterization is achieving feasibility at the single-patient level, systems biology approaches are likely to help discriminate alterations and developmentally related states of the cancer cell of

each individual tumor. These analyses would be extremely difficult using only the tools of statistical genomics, which require a much larger sample size. Although success stories are still limited, there is now a realistic, albeit cautious, expectation that these tools will allow a new level of personalized cancer care, based on the individual's genetic background. For instance, at least in cell lines, it has now been possible to partially predict sensitivity to specific compounds on the basis of their genetic landscape,[3] and biomarkers have been shown to be predictive of response and disease-free survival in neoadjuvant breast cancer treatment.[145]

Integrative analysis, a staple of the cancer systems biology approach, is now also starting to be adopted in clinical practice. For instance, although most pathology labs now perform single mutation screens to inform therapeutic choices (e.g., KRAS mutation screen in colorectal carcinomas), whole-genome sequencing is starting to be adopted for genotype-specific therapeutics,[146] and the identification of drug-accessible fusion events in a small number of patients is likely to become an additional tool for cancer therapeutics. In a pilot study, whole-genome, exome, and transcriptome sequencing have been performed in several patients with advanced cancer.[147] These sequencing data were integrated to detect cancer mutations such as structural rearrangement, copy number variation, and somatic point mutation and identify aberrant pathways that are driven by these mutations. Finally, distinct rationales for clinical trials of two patients were suggested according to their backgrounds of genetic mutations and pathway dysregulation.[147] This framework of personalized therapy development, which integrates extensive genetic sequencing, genomic profiling, and causal network inferring, is expected to be tested on a larger scale in the near future.

Finally, although most personalized-medicine approaches have focused on cancer heterogeneity across patients, tumor clones evolve according to fitness landscapes that are frequently determined by therapeutic choices, thus acquiring mutations that confer drug resistance.[148,149] For instance, a mathematical model was built to simulate the genetic dynamics and cell heterogeneity in a tumor.[150] Multiple personalized therapeutic strategies were tested by simulation using this model, and clinical outcomes were inferred. This simulation suggested that nonstandard personalized medicine, which takes into account subclones and dynamic genetic and epigenetic mutations, should substantially improve the clinical outcome after personalized therapeutics. Although this is just a theoretical simulation, it does imply that traditional personalized medicine based on the averaged and static genetic genomics may not be enough to tackle the complexity of tumor progression in individual patients. There is still a long way to go toward achieving a mature view of personalized medicine that benefits cancer patients to the largest degree. Yet, cancer systems biology, as an integrative and cross-disciplinary science, offers a novel and promising avenue of research in this direction.

Key References

1. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell*. 2006;9:157-173.
3. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-607.
6. Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17:98-110.
7. Compagno M, Lim WK, Grunn A, et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature*. 2009;459:717-721.
8. Taylor BS, Schultz N, Hieronymus H, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18:11-22.
9. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. *Nature*. 2009;461:747-753.
10. Narayanan M, Vetta A, Schadt EE, Zhu J. Simultaneous clustering of multiple gene expression and physical interaction datasets. *PLoS Comput Biol*. 2010;6:e1000742.
11. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 2011;144:27-40.
12. Califano A, Butte AJ, Friend S, Ideker T, Schadt E. Leveraging models of cell regulation and GWAS data in integrative network-based association studies. *Nat Genet*. 2012;44:841-847.
13. Schadt EE, Monks SA, Drake TA, et al. Genetics of gene expression surveyed in maize, mouse and man. *Nature*. 2003;422:297-302.
14. Yang X, Deignan JL, Qi H, et al. Validation of candidate causal genes for obesity that affect shared metabolic pathways and networks. *Nat Genet*. 2009;41:415-423.
15. Zhao X, D'Arca D, Lim WK, et al. The N-Myc-DLL3 cascade is suppressed by the ubiquitin ligase Huwe1 to inhibit proliferation and promote neurogenesis in the developing brain. *Dev Cell*. 2009;17:210-221.
16. Della Gatta G, Palomero T, Perez-Garcia A, et al. Reverse engineering of TLX oncogenic transcriptional networks identifies RUNX1 as tumor suppressor in T-ALL. *Nat Med*. 2012;18:436-440.
17. Sumazin P, Yang X, Chiu HS, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell*. 2011;147:370-381.
18. Kwong LN, Costello JC, Liu H, et al. Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat Med*. 2012;18:1503-1510.
19. Akavia UD, Litvin O, Kim J, et al. An integrated approach to uncover drivers of cancer. *Cell*. 2010;143:1005-1017.
20. Yarden Y, Pines G. The ERBB network: at last, cancer therapy meets systems biology. *Nat Rev Cancer*. 2012;12:553-563.
21. Schoeberl B, Pace EA, Fitzgerald JB, et al. Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis. *Sci Signal*. 2009;2 ra31.
22. De Keersmaecker K, Real PJ, Gatta GD, et al. The TLX1 oncogene drives aneuploidy in T cell transformation. *Nat Med*. 2010;16:1321-1327.
23. Wang K, Li M, Hakonarson H. Analysing biological pathways in genome-wide association studies. *Nat Rev Genet*. 2010;11:843-854.
24. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007;131:1190-1203.

25. Luscombe NM, Babu MM, Yu H, Snyder M, Teichmann SA, Gerstein M. Genomic analysis of regulatory network dynamics reveals large topological changes. *Nature*. 2004;431:308-312.
26. Wang K, Saito M, Bisikirska BC, et al. Genome-wide identification of post-translational modulators of transcription factor activity in human B cells. *Nat Biotechnol*. 2009;27:829-839.
27. Friedman N. Inferring cellular networks using probabilistic graphical models. *Science*. 2004;303:799-805.
30. TCGA-Consortium. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455: 1061-1068.
31. Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res*. 2011;39 (Database issue):D945–D950.
32. Larranaga P, Calvo B, Santana R, et al. Machine learning in bioinformatics. *Brief Bioinform*. 2006;7:86-112.
34. Hartemink AJ. Reverse engineering gene regulatory networks. *Nat Biotechnol*. 2005;23:554-555.
35. Sachs K, Perez O, Pe'er D, Lauffenburger DA, Nolan GP. Causal protein-signaling networks derived from multiparameter single-cell data. *Science*. 2005;308:523-529.
36. Andersson CR, Gustafsson MG, Strombergsson H. Quantitative chemogenomics: machine-learning models of protein-ligand interaction. *Curr Top Med Chem*. 2011;11:1978-1993.
37. Ezkurdia I, Bartoli L, Fariselli P, Casadio R, Valencia A, Tress ML. Progress and challenges in predicting protein-protein interaction sites. *Brief Bioinform*. 2009;10:233-246.
38. de Vries SJ, Bonvin AM. How proteins get in touch: interface prediction in the study of biomolecular complexes. *Curr Protein Pept Sci*. 2008;9: 394-406.
39. Franke V, Sikic M, Vlahovicek K. Prediction of interacting protein residues using sequence and structure data. *Methods Mol Biol*. 2012;819:233-251.
40. Lefebvre C, Rajbhandari P, Alvarez MJ, et al. A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol*. 2010;6:377.
42. Wu MC, Lin X. Prior biological knowledge-based approaches for the analysis of genome-wide expression profiles using gene sets and pathways. *Stat Methods Med Res*. 2009;18:577-593.
43. Ideker T, Dutkowski J, Hood L. Boosting signal-to-noise in complex biology: prior knowledge is power. *Cell*. 2011;144:860-863.
44. Marbach D, Costello JC, Kuffner R, et al. Wisdom of crowds for robust gene network inference. *Nat Methods*. 2012;9:796-804.
45. Zhang QC, Petrey D, Deng L, et al. Structure-based prediction of protein-protein interactions on a genome-wide scale. *Nature*. 2012;490: 556-560.
46. Bebek G, Koyuturk M, Price ND, Chance MR. Network biology methods integrating biological data for translational science. *Brief Bioinform*. 2012;13:446-459.
47. Lyles RH, Tang L, Lin J, Zhang Z, Mukherjee B. Likelihood-based methods for regression analysis with binary exposure status assessed by pooling. *Stat Med*. 2012;31:2485-2497.
48. Knott SA. Regression-based quantitative trait loci mapping: robust, efficient and effective. *Philos Trans R Soc Lond B Biol Sci*. 2005;360:1435-1442.
49. Li X, Yang T, Li S. Discrimination of serum Raman spectroscopy between normal and colorectal cancer using selected parameters and regression-discriminant analysis. *Appl Optics*. 2012;51:5038-5043.
50. Li X, Gill R, Cooper NG, Yoo JK, Datta S. Modeling microRNA-mRNA interactions using PLS regression in human colon cancer*BMC Med Genomics*. 2011;4:44 3123543.
51. Li H, Gui J. Partial Cox regression analysis for high-dimensional microarray gene expression data. *Bioinformatics*. 2004;20(suppl 1) i208-i115.
52. Sarder P, Schierding W, Cobb JP, Nehorai A. Estimating sparse gene regulatory networks using a Bayesian linear regression. *IEEE Trans Nanobiosci*. 2010;9:121-131.
53. Jin G, Fu C, Zhao H, Cui K, Chang J, Wong ST. A novel method of transcriptional response analysis to facilitate drug repositioning for cancer therapy. *Cancer Res*. 2012;72:33-44.
54. Zhang W, Chen Y, Sun F, Jiang R. DomainRBF: a Bayesian regression approach to the prioritization of candidate domains for complex diseases. *BMC Syst Biol*. 2011;5:55.
55. Jaqaman K, Danuser G. Linking data to models: data regression. *Nat Rev Mol Cell Biol*. 2006;7:813-819.
58. Waltermann C, Klipp E. Information theory based approaches to cellular signaling. *Biochim Biophys Acta*. 2011;1810:924-932.
59. Basso K, Margolin AA, Stolovitzky G, Klein U, Dalla-Favera R, Califano A. Reverse engineering of regulatory networks in human B cells. *Nat Genet*. 2005;37:382-390.
60. Goodarzi H, Najafabadi HS, Oikonomou P, et al. Systematic discovery of structural elements governing stability of mammalian messenger RNAs. *Nature*. 2012;485:264-268.
61. Drees BL, Thorsson V, Carter GW, et al. Derivation of genetic interaction networks from quantitative phenotype data. *Genome Biol*. 2005;6 R38.
62. Elemento O, Slonim N, Tavazoie S. A universal framework for regulatory element discovery across all genomes and data types. *Mol Cell*. 2007;28:337-350.
64. Wang K, Banerjee N, Margolin AA, Nemenman I, Califano A. Genome-wide discovery of modulators of transcriptional interactions in human B lymphocytes. *RECOMB 2006, also Lecture Notes Comp Sci*. 2006;3909:348-362.
65. Margolin AA, Nemenman I, Basso K, et al. ARACNe: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC Bioinformatics*. 2006;7(Suppl 1):S7.
66. Sumazin P, Yang X, Chiu HS, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell*. 2011;147:370-381.
67. Meyer PE, Lafitte F, Bontempi G. minet: A R/Bioconductor package for inferring large transcriptional networks using mutual information. *BMC Bioinformatics*. 2008;9:461.
68. Faith JJ, Hayete B, Thaden JT, et al. Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biol*. 2007;5:e8.
69. Pe'er D, Hacohen N. Principles and strategies for developing network models in cancer. *Cell*. 2011;144:864-873.
70. Lim WK, Lyashenko E, Califano A. Master regulators used as breast cancer metastasis classifier. *Pac Symp Biocomput*. 2009:504-515.
71. Carro MS, Lim WK, Alvarez MJ, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature*. 2010;463:318-325.
72. Wang K, Alvarez MJ, Bisikirska BC, et al. Dissecting the interface between signaling and transcriptional regulation in human B cells. *Pac Symp Biocomput*. 2009:264-275.
73. Bisikirska B, Adam SJ, Alvarez MJ, et al. STK38 is a critical upstream regulator of MYC's oncogenic activity in human B-cell lymphoma. *Oncogene*. 2012 Nov 26. doi: 10.1038/onc.2012.543. [Epub ahead of print].
74. Pang H, Hauser M, Minvielle S. Pathway-based identification of SNPs predictive of survival. *Eur J Hum Genet*. 2011;19:704-709.
75. Wegrzyn JL, Lee JM, Liechty J, Neale DB. PineSAP—sequence alignment and SNP identification pipeline. *Bioinformatics*. 2009;25:2609-2610.
76. Matukumalli LK, Grefenstette JJ, Hyten DL, Choi IY, Cregan PB, Van Tassell CP. Application of machine learning in SNP discovery. *BMC Bioinformatics*. 2006;7:4.
77. Ueki M, Tamiya G. Ultrahigh-dimensional variable selection method for whole-genome gene-gene interaction analysis. *BMC Bioinformatics*. 2012;13:72.
78. Cheng YK, Beroukhim R, Levine RL, Mellinghoff IK, Holland EC, Michor F. A mathematical methodology for determining the temporal order of pathway alterations arising during gliomagenesis. *PLoS Comput Biol*. 2012;8:e1002337.
79. Wong G, Leckie C, Kowalczyk AFSR. feature set reduction for scalable and accurate multi-class cancer subtype classification based on copy number. *Bioinformatics*. 2012;28:151-159.
80. Van Ness B, Ramos C, Haznadar M, et al. Genomic variation in myeloma: design, content, and initial application of the Bank On A Cure SNP Panel to detect associations with progression-free survival. *BMC Med*. 2008;6:26.
81. Han B, Park M, Chen XW. A Markov blanket-based method for detecting causal SNPs in GWAS. *BMC Bioinformatics*. 2010;11(Suppl 3):S5.
82. Yu K, Martin R, Rothman N, Zheng T, Lan Q. Two-sample comparison based on prediction error, with applications to candidate gene association studies. *Ann Hum Genet*. 2007;71(Pt 1):107-118.

83. Szymczak S, Biernacka JM, Cordell HJ, et al. Machine learning in genome-wide association studies. *Genet Epidemiol*. 2009;33(Suppl 1):S51-S57.
86. Schreiber SL, Shamji AF, Clemons PA, et al. Towards patient-based cancer therapeutics. *Nat Biotechnol*. 2010;28:904-906.
87. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
88. Franco-Zorrilla JM, Valli A, Todesco M, et al. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet*. 2007;39:1033-1037.
89. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature*. 2010;465:1033-1038.
90. Karreth FA, Tay Y, Perna D, et al. In vivo identification of tumor-suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. *Cell*. 2011;147:382-395.
91. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455:1061-1068.
92. Mailman MD, Feolo M, Jin Y, et al. The NCBI dbGaP database of genotypes and phenotypes. *Nat Genet*. 2007;39:1181-1186.
95. Keiser MJ, Setola V, Irwin JJ, et al. Predicting new molecular targets for known drugs. *Nature*. 2009;462:175-181.
96. Campillos M, Kuhn M, Gavin AC, Jensen LJ, Bork P. Drug target identification using side-effect similarity. *Science*. 2008;321:263-266.
97. di Bernardo D, Thompson MJ, Gardner TS, et al. Chemogenomic profiling on a genome-wide scale using reverse-engineered gene networks. *Nat Biotechnol*. 2005;23:377-383.
98. Mani KM, Lefebvre C, Wang K, et al. A systems biology approach to prediction of oncogenes and molecular perturbation targets in B-cell lymphomas. *Mol Syst Biol*. 2008;4:169.
99. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science*. 2006;313:1929-1935.
100. Chen BJ, Causton HC, Mancenido D, Goddard NL, Perlstein EO, Pe'er D. Harnessing gene expression to identify the genetic basis of drug resistance. *Mol Syst Biol*. 2009;5:310.
101. Iorio F, Bosotti R, Scacheri E, et al. Discovery of drug mode of action and drug repositioning from transcriptional responses. *Proc Natl Acad Sci U S A*. 2010;107:14621-14626.
102. Lee MJ, Ye AS, Gardino AK, et al. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell*. 2012;149:780-794.
105. Califano A, Butte A, Friend SH, Ideker T, Schadt EE. Integrative Network-based Association Studies: Leveraging cell regulatory models in the post-GWAS era. *Nat Precedings*. 2011. http://precedings.nature.com/documents/5732/version/1.
106. Schadt EE, Lamb J, Yang X, et al. An integrative genomics approach to infer causal associations between gene expression and disease. *Nat Genet*. 2005;37:710-717.
107. Konig R, Stertz S, Zhou Y, et al. Human host factors required for influenza virus replication. *Nature*. 2010;463:813-817.
108. Zhu J, Zhang B, Smith EN, et al. Integrating large-scale functional genomic data to dissect the complexity of yeast regulatory networks. *Nat Genet*. 2008;40:854-861.
109. Nijman SM. Synthetic lethality: general principles, utility and detection using genetic screens in human cells. *FEBS Lett*. 2011;585:1-6.
110. Mullenders J, Bernards R. Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer. *Oncogene*. 2009;28:4409-4420.
111. Boutros M, Ahringer J. The art and design of genetic screens: RNA interference. *Nat Rev Genet*. 2008;9:554-566.
112. Amit I, Garber M, Chevrier N, et al. Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. *Science*. 2009;326:257-263.
113. Silva JM, Marran K, Parker JS, et al. Profiling essential genes in human mammary cells by multiplex RNAi screening. *Science*. 2008;319:617-620.
114. Schlabach MR, Luo J, Solimini NL, et al. Cancer proliferation gene discovery through functional genomics. *Science*. 2008;319:620-624.
115. Luo B, Cheung HW, Subramanian A, et al. Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci U S A*. 2008;105:20380-20385.
116. Holzel M, Huang S, Koster J, et al. NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome. *Cell*. 2010;142:218-229.
117. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 2011;470:115-119.
118. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q– syndrome gene by RNA interference screen. *Nature*. 2008;451:335-339.
119. Whitehurst AW, Bodemann BO, Cardenas J, et al. Synthetic lethal screen identification of chemosensitizer loci in cancer cells. *Nature*. 2007;446:815-819.
120. Neumann B, Held M, Liebel U, et al. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat Methods*. 2006;3:385-390.
121. Jones TR, Carpenter AE, Lamprecht MR, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A*. 2009;106:1826-1831.
122. Mukherji M, Bell R, Supekova L, et al. Genome-wide functional analysis of human cell-cycle regulators. *Proc Natl Acad Sci U S A*. 2006;103:14819-14824.
123. Cheung HW, Cowley GS, Weir BA, et al. Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci U S A*. 2011;108:12372-12377.
124. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta*. 2010;1805:105-117.
125. Cai L, Friedman N, Xie XS. Stochastic protein expression in individual cells at the single molecule level. *Nature*. 2006;440:358-362.
126. Trott J, Hayashi K, Surani A, Babu MM, Martinez-Arias A. Dissecting ensemble networks in ES cell populations reveals micro-heterogeneity underlying pluripotency. *Mol Biosyst*. 2012;8:744-752.
127. Mantzaris NV. From single-cell genetic architecture to cell population dynamics: quantitatively decomposing the effects of different population heterogeneity sources for a genetic network with positive feedback architecture. *Biophys J*. 2007;92:4271-4288.
128. Cohen AA, Geva-Zatorsky N, Eden E, et al. Dynamic proteomics of individual cancer cells in response to a drug. *Science*. 2008;322:1511-1516.
129. Toriello NM, Douglas ES, Thaitrong N, et al. Integrated microfluidic bioprocessor for single-cell gene expression analysis. *Proc Natl Acad Sci U S A*. 2008;105:20173-20178.
130. Hou Y, Song L, Zhu P, et al. Single-cell exome sequencing and monoclonal evolution of a JAK2-negative myeloproliferative neoplasm. *Cell*. 2012;148:873-885.
131. Xu X, Hou Y, Yin X, et al. Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. *Cell*. 2012;148:886-895.
132. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell*.2004;118 2172-2128.
133. Ornatsky O, Bandura D, Baranov V, Nitz M, Winnik MA, Tanner S. Highly multiparametric analysis by mass cytometry. *J Immunol Methods*. 2010;361:1-20.
134. Behbehani GK, Bendall SC, Clutter MR, Fantl WJ, Nolan GP. Single-cell mass cytometry adapted to measurements of the cell cycle. *Cytometry A*. 2012;81:552-566.
135. Whitesides GM. The origins and the future of microfluidics. *Nature*. 2006;442:368-373.
136. Taniguchi K, Kajiyama T, Kambara H. Quantitative analysis of gene expression in a single cell by qPCR. *Nat Methods*. 2009;6:503-506.
137. Marcus JS, Anderson WF, Quake SR. Microfluidic single-cell mRNA isolation and analysis. *Anal Chem*. 2006;78:3084-3089.
138. Tang F, Barbacioru C, Wang Y, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods*. 2009;6:377-382.
139. Heinemann M, Zenobi R. Single cell metabolomics. *Curr Opin Biotechnol*. 2011;22:26-31.
140. Wang D, Bodovitz S. Single cell analysis: the new frontier in "omics." *Trends Biotechnol*. 2010;28:281-290.
141. Heiser LM, Sadanandam A, Kuo WL, et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:2724-2729.

142. Collisson EA, Sadanandam A, Olson P, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med*. 2011;17:500-503.
143. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486:346-352.
144. Choi N, Zhang B, Zhang L, Ittmann M, Xin L. Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. *Cancer Cell*. 2012;21:253-265.
145. Esserman LJ, Berry DA, Cheang MC, et al. Chemotherapy response and recurrence-free survival in neoadjuvant breast cancer depends on biomarker profiles: results from the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657). *Breast Cancer Res Treatment*. 2012;132:1049-1062.
146. Leary RJ, Kinde I, Diehl F, et al. Development of personalized tumor biomarkers using massively parallel sequencing. *Sci Transl Med*. 2010;2 20ra14.
147. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study*Sci Transl Med*. 2011;3 111ra21.
149. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807-1812.
150. Beckman RA, Schemmann GS, Yeang CH. Impact of genetic dynamics and single-cell heterogeneity on development of nonstandard personalized medicine strategies for cancer. *Proc Natl Acad Sci U S A*. 2012 Sep 4;109(36):14586-91. doi: 10.1073/pnas.1203559109. Epub 2012 Aug 13.

References

1. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell*. 2006;9:157-173.
2. Lossos IS, Alizadeh AA, Diehn M, et al. Transformation of follicular lymphoma to diffuse large-cell lymphoma: alternative patterns with increased or decreased expression of c-myc and its regulated genes. *Proc Natl Acad Sci U S A*. 2002;99:8886-8891.
3. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-607.
4. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*. 2000;403:503-511.
5. Klein U, Tu Y, Stolovitzky GA, et al. Transcriptional analysis of the B cell germinal center reaction. *Proc Natl Acad Sci U S A*. 2003;100:2639-2644.
6. Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17:98-110.
7. Compagno M, Lim WK, Grunn A, et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature*. 2009;459:717-721.
8. Taylor BS, Schultz N, Hieronymus H, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18:11-22.
9. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. *Nature*. 2009;461:747-753.
10. Narayanan M, Vetta A, Schadt EE, Zhu J. Simultaneous clustering of multiple gene expression and physical interaction datasets. *PLoS Comput Biol*. 2010;6:e1000742.
11. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 2011;144:27-40.
12. Califano A, Butte AJ, Friend S, Ideker T, Schadt E. Leveraging models of cell regulation and GWAS data in integrative network-based association studies. *Nat Genet*. 2012;44:841-847.
13. Schadt EE, Monks SA, Drake TA, et al. Genetics of gene expression surveyed in maize, mouse and man. *Nature*. 2003;422:297-302.
14. Yang X, Deignan JL, Qi H, et al. Validation of candidate causal genes for obesity that affect shared metabolic pathways and networks. *Nat Genet*. 2009;41:415-423.
15. Zhao X, D'Arca D, Lim WK, et al. The N-Myc-DLL3 cascade is suppressed by the ubiquitin ligase Huwe1 to inhibit proliferation and promote neurogenesis in the developing brain. *Dev Cell*. 2009;17:210-221.
16. Della Gatta G, Palomero T, Perez-Garcia A, et al. Reverse engineering of TLX oncogenic transcriptional networks identifies RUNX1 as tumor suppressor in T-ALL. *Nat Med*. 2012;18:436-440.
17. Sumazin P, Yang X, Chiu HS, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell*. 2011;147:370-381.
18. Kwong LN, Costello JC, Liu H, et al. Oncogenic NRAS signaling differentially regulates survival and proliferation in melanoma. *Nat Med*. 2012;18:1503-1510.
19. Akavia UD, Litvin O, Kim J, et al. An integrated approach to uncover drivers of cancer. *Cell*. 2010;143:1005-1017.
20. Yarden Y, Pines G. The ERBB network: at last, cancer therapy meets systems biology. *Nat Rev Cancer*. 2012;12:553-563.
21. Schoeberl B, Pace EA, Fitzgerald JB, et al. Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis. *Sci Signal*. 2009;2 ra31.
22. De Keersmaecker K, Real PJ, Gatta GD, et al. The TLX1 oncogene drives aneuploidy in T cell transformation. *Nat Med*. 2010;16:1321-1327.
23. Wang K, Li M, Hakonarson H. Analysing biological pathways in genome-wide association studies. *Nat Rev Genet*. 2010;11:843-854.
24. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007;131:1190-1203.
25. Luscombe NM, Babu MM, Yu H, Snyder M, Teichmann SA, Gerstein M. Genomic analysis of regulatory network dynamics reveals large topological changes. *Nature*. 2004;431:308-312.
26. Wang K, Saito M, Bisikirska BC, et al. Genome-wide identification of post-translational modulators of transcription factor activity in human B cells. *Nat Biotechnol*. 2009;27:829-839.
27. Friedman N. Inferring cellular networks using probabilistic graphical models. *Science*. 2004;303:799-805.
28. Gardner TS, di Bernardo D, Lorenz D, Collins JJ. Inferring genetic networks and identifying compound mode of action via expression profiling. *Science*. 2003;301:102-105.
29. Tavazoie S, Hughes JD, Campbell MJ, Cho RJ, Church GM. Systematic determination of genetic network architecture. *Nat Genet*. 1999;22:281-285.
30. TCGA-Consortium. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455:1061-1068.
31. Forbes SA, Bindal N, Bamford S, et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res*. 2011;39 (Database issue):D945–D950.
32. Larranaga P, Calvo B, Santana R, et al. Machine learning in bioinformatics. *Brief Bioinform*. 2006;7:86-112.
33. Segal E, Shapira M, Regev A, et al. Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. *Nat Genet*. 2003;34:166-176.
34. Hartemink AJ. Reverse engineering gene regulatory networks. *Nat Biotechnol*. 2005;23:554-555.
35. Sachs K, Perez O, Pe'er D, Lauffenburger DA, Nolan GP. Causal protein-signaling networks derived from multiparameter single-cell data. *Science*. 2005;308:523-529.
36. Andersson CR, Gustafsson MG, Strombergsson H. Quantitative chemogenomics: machine-learning models of protein-ligand interaction. *Curr Top Med Chem*. 2011;11:1978-1993.
37. Ezkurdia I, Bartoli L, Fariselli P, Casadio R, Valencia A, Tress ML. Progress and challenges in predicting protein-protein interaction sites. *Brief Bioinform*. 2009;10:233-246.
38. de Vries SJ, Bonvin AM. How proteins get in touch: interface prediction in the study of biomolecular complexes. *Curr Protein Pept Sci*. 2008;9:394-406.
39. Franke V, Sikic M, Vlahovicek K. Prediction of interacting protein residues using sequence and structure data. *Methods Mol Biol*. 2012;819:233-251.
40. Lefebvre C, Rajbhandari P, Alvarez MJ, et al. A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol*. 2010;6:377.
41. Jansen R, Yu H, Greenbaum D, et al. A Bayesian networks approach for predicting protein-protein interactions from genomic data. *Science*. 2003;302:449-453.
42. Wu MC, Lin X. Prior biological knowledge-based approaches for the analysis of genome-wide expression profiles using gene sets and pathways. *Stat Methods Med Res*. 2009;18:577-593.
43. Ideker T, Dutkowski J, Hood L. Boosting signal-to-noise in complex biology: prior knowledge is power. *Cell*. 2011;144:860-863.
44. Marbach D, Costello JC, Kuffner R, et al. Wisdom of crowds for robust gene network inference. *Nat Methods*. 2012;9:796-804.
45. Zhang QC, Petrey D, Deng L, et al. Structure-based prediction of protein-protein interactions on a genome-wide scale. *Nature*. 2012;490:556-560.
46. Bebek G, Koyuturk M, Price ND, Chance MR. Network biology methods integrating biological data for translational science. *Brief Bioinform*. 2012;13:446-459.
47. Lyles RH, Tang L, Lin J, Zhang Z, Mukherjee B. Likelihood-based methods for regression analysis with binary exposure status assessed by pooling. *Stat Med*. 2012;31:2485-2497.
48. Knott SA. Regression-based quantitative trait loci mapping: robust, efficient and effective. *Philos Trans R Soc Lond B Biol Sci*. 2005;360:1435-1442.
49. Li X, Yang T, Li S. Discrimination of serum Raman spectroscopy between normal and colorectal cancer using selected parameters and regression-discriminant analysis. *Appl Optics*. 2012;51:5038-5043.
50. Li X, Gill R, Cooper NG, Yoo JK, Datta S. Modeling microRNA-mRNA interactions using PLS regression in human colon cancer. *BMC Med Genomics*. 2011;4:44.
51. Li H, Gui J. Partial Cox regression analysis for high-dimensional microarray gene expression data.*Bioinformatics*. 2004;20(Suppl 1):i208-i115.

52. Sarder P, Schierding W, Cobb JP, Nehorai A. Estimating sparse gene regulatory networks using a Bayesian linear regression. *IEEE Trans Nanobiosci*. 2010;9:121-131.
53. Jin G, Fu C, Zhao H, Cui K, Chang J, Wong ST. A novel method of transcriptional response analysis to facilitate drug repositioning for cancer therapy. *Cancer Res*. 2012;72:33-44.
54. Zhang W, Chen Y, Sun F, Jiang R. DomainRBF: a Bayesian regression approach to the prioritization of candidate domains for complex diseases. *BMC Syst Biol*. 2011;5:55.
55. Jaqaman K, Danuser G. Linking data to models: data regression. *Nat Rev Mol Cell Biol*. 2006;7:813-819.
56. Yeung MK, Tegner J, Collins JJ. Reverse engineering gene networks using singular value decomposition and robust regression. *Proc Natl Acad Sci U S A*. 2002;99:6163-6168.
57. Shannon CE. A mathematical theory of communication. *At&T Tech J*. 1948;27:379-423.
58. Waltermann C, Klipp E. Information theory based approaches to cellular signaling. *Biochim Biophys Acta*. 2011;1810:924-932.
59. Basso K, Margolin AA, Stolovitzky G, Klein U, Dalla-Favera R, Califano A. Reverse engineering of regulatory networks in human B cells. *Nat Genet*. 2005;37:382-390.
60. Goodarzi H, Najafabadi HS, Oikonomou P, et al. Systematic discovery of structural elements governing stability of mammalian messenger RNAs. *Nature*. 2012;485:264-268.
61. Drees BL, Thorsson V, Carter GW, et al. Derivation of genetic interaction networks from quantitative phenotype data.*Genome Biol*. 2005;6 R38.
62. Elemento O, Slonim N, Tavazoie S. A universal framework for regulatory element discovery across all genomes and data types. *Mol Cell*. 2007;28:337-350.
63. Steuer R, Kurths J, Daub CO, Weise J, Selbig J. The mutual information: detecting and evaluating dependencies between variables. *Bioinformatics*. 2002;18(Suppl 2):S231-S240.
64. Wang K, Banerjee N, Margolin AA, Nemenman I, Califano A. Genome-wide discovery of modulators of transcriptional interactions in human B lymphocytes. *RECOMB 2006, also Lecture Notes Comp Sci*. 2006;3909:348-362.
65. Margolin AA, Nemenman I, Basso K, et al. ARACNe: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC Bioinformatics*. 2006(7 Suppl 1):S7.
66. Sumazin P, Yang X, Chiu HS, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell*. 2011;147:370-381.
67. Meyer PE, Lafitte F, Bontempi G. minet: A R/Bioconductor package for inferring large transcriptional networks using mutual information. *BMC Bioinformatics*. 2008;9:461.
68. Faith JJ, Hayete B, Thaden JT, et al. Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biol*. 2007;5:e8.
69. Pe'er D, Hacohen N. Principles and strategies for developing network models in cancer. *Cell*. 2011;144:864-873.
70. Lim WK, Lyashenko E, Califano A. Master regulators used as breast cancer metastasis classifier. *Pac Symp Biocomput*. 2009:504-515.
71. Carro MS, Lim WK, Alvarez MJ, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature*. 2010;463:318-325.
72. Wang K, Alvarez MJ, Bisikirska BC, et al. Dissecting the interface between signaling and transcriptional regulation in human B cells. *Pac Symp Biocomput*. 2009:264-275.
73. Bisikirska B, Adam SJ, Alvarez MJ, et al. STK38 is a critical upstream regulator of MYC's oncogenic activity in human B-cell lymphoma. *Oncogene*. 2012 Nov 26. doi: 10.1038/onc.2012.543. [Epub ahead of print].
74. Pang H, Hauser M, Minvielle S. Pathway-based identification of SNPs predictive of survival. *Eur J Hum Genet*. 2011;19:704-709.
75. Wegrzyn JL, Lee JM, Liechty J, Neale DB. PineSAP—sequence alignment and SNP identification pipeline. *Bioinformatics*. 2009;25:2609-2610.
76. Matukumalli LK, Grefenstette JJ, Hyten DL, Choi IY, Cregan PB, Van Tassell CP. Application of machine learning in SNP discovery. *BMC Bioinformatics*. 2006;7:4.
77. Ueki M, Tamiya G. Ultrahigh-dimensional variable selection method for whole-genome gene-gene interaction analysis. *BMC Bioinformatics*. 2012;13:72.
78. Cheng YK, Beroukhim R, Levine RL, Mellinghoff IK, Holland EC, Michor F. A mathematical methodology for determining the temporal order of pathway alterations arising during gliomagenesis. *PLoS Comput Biol*. 2012;8:e1002337.
79. Wong G, Leckie C, Kowalczyk AFSR. feature set reduction for scalable and accurate multi-class cancer subtype classification based on copy number. *Bioinformatics*. 2012;28:151-159.
80. Van Ness B, Ramos C, Haznadar M, et al. Genomic variation in myeloma: design, content, and initial application of the Bank On A Cure SNP Panel to detect associations with progression-free survival. *BMC Med*. 2008;6:26.
81. Han B, Park M, XW Chen. A Markov blanket-based method for detecting causal SNPs in GWAS. *BMC Bioinformatics*. 2010;11(Suppl 3):S5.
82. Yu K, Martin R, Rothman N, Zheng T, Lan Q. Two-sample comparison based on prediction error, with applications to candidate gene association studies. *Ann Hum Genet*. 2007;71(Pt 1):107-118.
83. Szymczak S, Biernacka JM, Cordell HJ, et al. Machine learning in genome-wide association studies. *Genet Epidemiol*. 2009;33(Suppl 1):S51-S57.
84. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med*. 2001;194:1861-1874.
85. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heel of cancer. *Science*. 2002;297:63-64.
86. Schreiber SL, Shamji AF, Clemons PA, et al. Towards patient-based cancer therapeutics. *Nat Biotechnol*. 2010;28:904-906.
87. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
88. Franco-Zorrilla JM, Valli A, Todesco M, et al. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet*. 2007;39:1033-1037.
89. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature*. 2010;465:1033-1038.
90. Karreth FA, Tay Y, Perna D, et al. In vivo identification of tumor-suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. *Cell*. 2011;147:382-395.
91. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455:1061-1068.
92. Mailman MD, Feolo M, Jin Y, et al. The NCBI dbGaP database of genotypes and phenotypes. *Nat Genet*. 2007;39:1181-1186.
93. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-1037.
94. Croom KF, Perry CM. Imatinib mesylate: in the treatment of gastrointestinal stromal tumours. *Drugs*. 2003;63:513-522; discussion 23–24.
95. Keiser MJ, Setola V, Irwin JJ, et al. Predicting new molecular targets for known drugs. *Nature*. 2009;462:175-181.
96. Campillos M, Kuhn M, Gavin AC, Jensen LJ, Bork P. Drug target identification using side-effect similarity. *Science*. 2008;321:263-266.
97. di Bernardo D, Thompson MJ, Gardner TS, et al. Chemogenomic profiling on a genome-wide scale using reverse-engineered gene networks. *Nat Biotechnol*. 2005;23:377-383.
98. Mani KM, Lefebvre C, Wang K, et al. A systems biology approach to prediction of oncogenes and molecular perturbation targets in B-cell lymphomas. *Mol Syst Biol*. 2008;4:169.
99. Lamb J, Crawford ED, Peck D, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science*. 2006;313:1929-1935.
100. Chen BJ, Causton HC, Mancenido D, Goddard NL, Perlstein EO, Pe'er D. Harnessing gene expression to identify the genetic basis of drug resistance. *Mol Syst Biol*. 2009;5:310.
101. Iorio F, Bosotti R, Scacheri E, et al. Discovery of drug mode of action and drug repositioning from transcriptional responses. *Proc Natl Acad Sci U S A*. 2010;107:14621-14626.
102. Lee MJ, Ye AS, Gardino AK, et al. Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell*. 2012;149:780-794.
103. Kitano H. Computational systems biology. *Nature*. 2002;420:206-210.
104. Ideker T, Galitski T, Hood L. A new approach to decoding life: systems biology. *Annu Rev Genomics Hum Genet*. 2001;2:343-372.

105. Califano A, Butte A, Friend SH, Ideker T, Schadt EE. Integrative Network-based Association Studies: Leveraging cell regulatory models in the post-GWAS era. *Nat Proceedings*. 2011. http://precedings.nature.com/documents/5732/version/1.
106. Schadt EE, Lamb J, Yang X, et al. An integrative genomics approach to infer causal associations between gene expression and disease. *Nat Genet*. 2005;37:710-717.
107. Konig R, Stertz S, Zhou Y, et al. Human host factors required for influenza virus replication. *Nature*. 2010;463:813-817.
108. Zhu J, Zhang B, Smith EN, et al. Integrating large-scale functional genomic data to dissect the complexity of yeast regulatory networks. *Nat Genet*. 2008;40:854-861.
109. Nijman SM. Synthetic lethality: general principles, utility and detection using genetic screens in human cells. *FEBS Lett*. 2011;585:1-6.
110. Mullenders J, Bernards R. Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer. *Oncogene*. 2009;28:4409-4420.
111. Boutros M, Ahringer J. The art and design of genetic screens: RNA interference. *Nat Rev Genet*. 2008;9:554-566.
112. Amit I, Garber M, Chevrier N, et al. Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. *Science*. 2009;326:257-263.
113. Silva JM, Marran K, Parker JS, et al. Profiling essential genes in human mammary cells by multiplex RNAi screening. *Science*. 2008;319:617-620.
114. Schlabach MR, Luo J, Solimini NL, et al. Cancer proliferation gene discovery through functional genomics. *Science*. 2008;319:620-624.
115. Luo B, Cheung HW, Subramanian A, et al. Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci U S A*. 2008;105:20380-20385.
116. Holzel M, Huang S, Koster J, et al. NF1 is a tumor suppressor in neuroblastoma that determines retinoic acid response and disease outcome. *Cell*. 2010;142:218-229.
117. Ngo VN, Young RM, Schmitz R, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 2011;470:115-119.
118. Ebert BL, Pretz J, Bosco J, et al. Identification of RPS14 as a 5q– syndrome gene by RNA interference screen. *Nature*. 2008;451:335-339.
119. Whitehurst AW, Bodemann BO, Cardenas J, et al. Synthetic lethal screen identification of chemosensitizer loci in cancer cells. *Nature*. 2007;446:815-819.
120. Neumann B, Held M, Liebel U, et al. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat Methods*. 2006;3:385-390.
121. Jones TR, Carpenter AE, Lamprecht MR, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A*. 2009;106:1826-1831.
122. Mukherji M, Bell R, Supekova L, et al. Genome-wide functional analysis of human cell-cycle regulators. *Proc Natl Acad Sci U S A*. 2006;103:14819-14824.
123. Cheung HW, Cowley GS, Weir BA, et al. Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci U S A*. 2011;108:12372-12377.
124. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta*. 2010;1805:105-117.
125. Cai L, Friedman N, Xie XS. Stochastic protein expression in individual cells at the single molecule level. *Nature*. 2006;440:358-362.
126. Trott J, Hayashi K, Surani A, Babu MM, Martinez-Arias A. Dissecting ensemble networks in ES cell populations reveals micro-heterogeneity underlying pluripotency. *Mol Biosyst*. 2012;8:744-752.
127. Mantzaris NV. From single-cell genetic architecture to cell population dynamics: quantitatively decomposing the effects of different population heterogeneity sources for a genetic network with positive feedback architecture. *Biophys J*. 2007 Jun 15;92(12):4271-88. Epub 2007 Mar 23.
128. Cohen AA, Geva-Zatorsky N, Eden E, et al. Dynamic proteomics of individual cancer cells in response to a drug. *Science*. 2008;322:1511-1516.
129. Toriello NM, Douglas ES, Thaitrong N, et al. Integrated microfluidic bioprocessor for single-cell gene expression analysis. *Proc Natl Acad Sci U S A*. 2008;105:20173-20178.
130. Hou Y, Song L, Zhu P, et al. Single-cell exome sequencing and monoclonal evolution of a JAK2-negative myeloproliferative neoplasm. *Cell*. 2012;148:873-885.
131. Xu X, Hou Y, Yin X, et al. Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor. *Cell*. 2012;148:886-895.
132. Irish JM, Hovland R, Krutzik PO, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell*. 2004;118 2172-2128.
133. Ornatsky O, Bandura D, Baranov V, Nitz M, Winnik MA, Tanner S. Highly multiparametric analysis by mass cytometry. *J Immunol Methods*. 2010;361:1-20.
134. Behbehani GK, Bendall SC, Clutter MR, Fantl WJ, Nolan GP. Single-cell mass cytometry adapted to measurements of the cell cycle. *Cytometry A*. 2012;81:552-566.
135. Whitesides GM. The origins and the future of microfluidics. *Nature*. 2006;442:368-373.
136. Taniguchi K, Kajiyama T, Kambara H. Quantitative analysis of gene expression in a single cell by qPCR. *Nat Methods*. 2009;6:503-506.
137. Marcus JS, Anderson WF, Quake SR. Microfluidic single-cell mRNA isolation and analysis. *Anal Chem*. 2006;78:3084-3089.
138. Tang F, Barbacioru C, Wang Y, et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nat Methods*. 2009;6:377-382.
139. Heinemann M, Zenobi R. Single cell metabolomics. *Curr Opin Biotechnol*. 2011;22:26-31.
140. Wang D, Bodovitz S. Single cell analysis: the new frontier in "omics." *Trends Biotechnol*. 2010;28:281-290.
141. Heiser LM, Sadanandam A, Kuo WL, et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:2724-2729.
142. Collisson EA, Sadanandam A, Olson P, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med*. 2011;17:500-503.
143. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486:346-352.
144. Choi N, Zhang B, Zhang L, Ittmann M, Xin L. Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. *Cancer Cell*. 2012;21:253-265.
145. Esserman LJ, Berry DA, Cheang MC, et al. Chemotherapy response and recurrence-free survival in neoadjuvant breast cancer depends on biomarker profiles: results from the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657). *Breast Cancer Res Treatment*. 2012;132:1049-1062.
146. Leary RJ, Kinde I, Diehl F, et al. Development of personalized tumor biomarkers using massively parallel sequencing. *Sci Transl Med*. 2010;2 20ra14.
147. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. *Sci Transl Med*. 2011;3 111ra21.
148. Loeb LA, Springgate CF, Battula N. Errors in DNA replication as a basis of malignant changes. *Cancer Res*. 1974;34:2311-2321.
149. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807-1812.
150. Beckman RA, Schemmann GS, Yeang CH. Impact of genetic dynamics and single-cell heterogeneity on development of nonstandard personalized medicine strategies for cancer. *Proc Natl Acad Sci U S A*. 2012.

Xifeng Wu and Jian Gu

21

Biomarkers for Assessing Risk of Cancer

Cancer is a complex disease involving both environmental and genetic determinants. The majority of cancers are caused by environmental and lifestyle factors, including smoking, alcohol use, infectious agents, occupation, diet, obesity, and lack of physical activity. However, only a small number of cancers, such as lung cancer (smoking) and cervical cancer (human papillomavirus [HPV] infection), have a major environmental risk factor that accounts for the bulk of disease in the population. For most cancers, the exposures have weak effects, and how to better assess the exposures and their effects remains a challenge. On the other hand, even for those cancers with a predominant environmental risk factor, only a small fraction of exposed individuals develop the cancer. For example, it is estimated that only 1 in 10 smokers develops lung cancer, and the rate of developing cervical cancer in high-risk carriers of HPV is even lower. Our understanding of the etiology of cancer in terms of environmental factors and genetic susceptibility is still rather limited, and the interplay among these etiological constituents is poorly understood. In recent years, biomarkers have been playing an increasingly important role in cancer etiological study to help better determine exposures, evaluate effects, and assess susceptibility.[1-3]

Figure 21-1 shows the spectrum of biomarkers along the continuum of carcinogenic process from environmental exposure to cancer development. Biomarkers of cancer etiology can be classified into three broad categories: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility.[1-4] Biomarkers of exposure indicate the presence of a carcinogenic compound or its biological interactions with cellular molecules, which can further be categorized into biomarkers of internal dose (e.g., carcinogens or their metabolites in bodily fluids, and circulating antibodies to infectious agents) and biomarkers of biologically effective dose (e.g., DNA and protein adducts).[5] Biomarkers of effect are biological indicators of the body's response to exposure and reflect the interaction between exposure and the human body. Biomarkers of effect encompass a broad array of early biological responses and altered structure and function of cells and tissues, such as chromosomal instability, gene mutation, epigenetic alteration (DNA methylation, histone modification, chromatin remodeling, and microRNA expression), changes in mRNA transcription and protein expression, and altered cell structure and function. Biomarkers of susceptibility (cancer risk) can be derived from each of the steps along the continuum of the carcinogenic process, reflecting interindividual variations in absorbing, distributing, metabolizing (activating and detoxifying), and excreting carcinogens; sensitivity to formation of macromolecule (DNA and protein) adducts; and ability to repair these adducts and eliminate damaged and premalignant cells. This categorization, however, is somewhat arbitrary, and the distinction among the three categories of biomarkers could be blurred. For instance, some chromosome instability biomarkers (e.g., micronuclei and chromosome aberrations) can be considered markers of effect as well as markers of susceptibility, because these markers not only indicate the early biological effect of exposure, but also reflect an individual's ability to metabolize carcinogens and repair DNA damage. Moreover, these markers have certain degrees of genetic heritability and can predict the risk of cancer independent of exposure level. Therefore, the interplay among these three categories of biomarkers is crucial for a complete understanding of cancer etiology. In the next few sections, we highlight some examples of biomarkers of exposure and biomarkers of effect. We also describe in more detail our current knowledge of biomarkers of cancer susceptibility.

Biomarkers of Exposure

Biomarkers of exposure can provide the most direct evidence of human exposure to a carcinogen as well as internal dose and biologically effective dose, affirm exposure-cancer associations established from traditional epidemiological approaches, and provide biological plausibility for the observed exposure-cancer association.

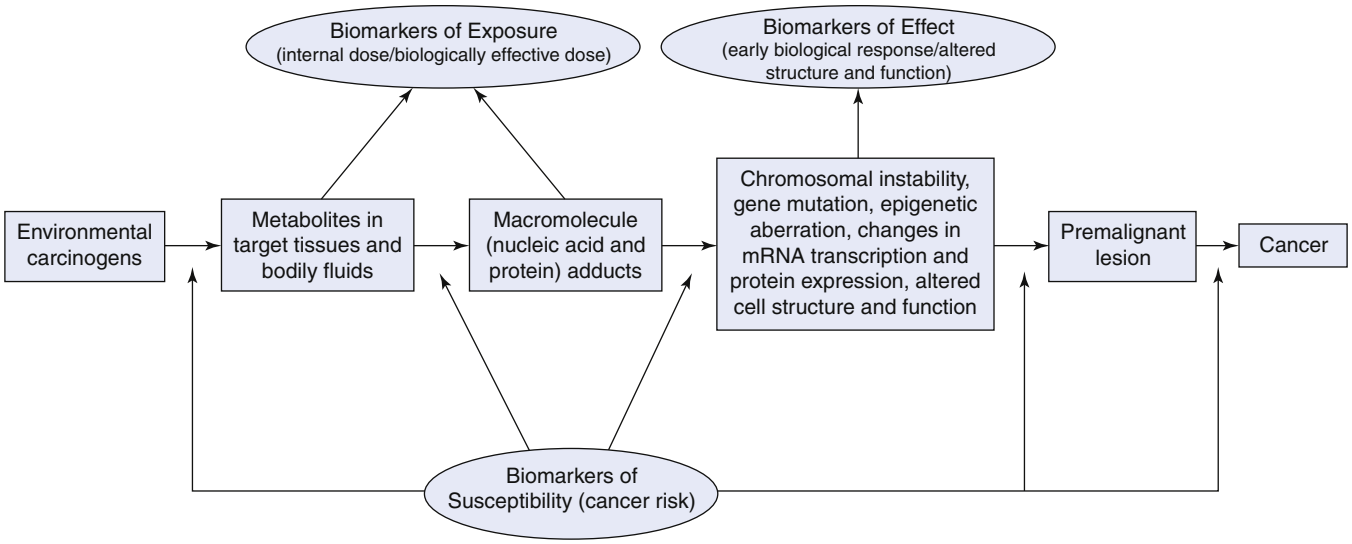


Figure 21-1 Biomarkers in cancer etiology Biomarkers in cancer etiologic studies can be classified into three broad categories: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. Biomarkers of susceptibility (cancer risk) can be derived from each of the steps along the continuum of the carcinogenic process, reflecting interindividual variations in absorption, distribution, metabolism (activating and detoxifying), and excretion of carcinogens; sensitivity to formation of macromolecule (DNA and protein) adducts; and ability to repair macromolecule damage, restore normal cellular functions, and eliminate premalignant cells.

Biomarkers of Internal Dose

Biomarkers of internal dose measure levels of a carcinogen or its metabolite in human tissues, bodily fluids, and excreta.[5] These biomarkers are not bound to cellular targets but provide a measure of exposure, absorption, metabolism, and excretion. There is generally a good correlation between external exposure and internal dose; however, the involvement of absorption and metabolism and interindividual variation in these processes suggest that the relationship may not always be simple. One of the classical examples of validated biomarkers of internal dose that contributed greatly to elucidate the environmental cause of human cancer is urinary aflatoxin and its metabolites. Aflatoxins have long been suspected to be human hepatic carcinogens, but the strongest evidence came from a prospective nested case-control study in which the authors measured urinary aflatoxin B1 (AFB1), its metabolites AFP1 and AFM1, and DNA adducts (AFB1-N7-Guanine) to assess the relation between aflatoxin exposure and liver cancer. Subjects with liver cancer were more likely to have detectable concentrations of any of the aflatoxin metabolites than controls, and the highest relative risk was for AFP1 (6.2-fold). Moreover, there was a strong interaction between chronic hepatitis B infection and aflatoxin exposure in liver cancer risk.[6] Tobacco-specific metabolites are the most studied biomarkers of internal dose. Cotinine is the main metabolite of nicotine, and the measurement of serum/plasma cotinine offers higher accuracy than self-reports in assessing tobacco smoking.[7] Two prospective studies have reported that higher levels of serum cotinine[8] and urinary cotinine[9] were associated with higher risk of lung cancer. The risk estimate of tobacco smoking and lung cancer from serum cotinine might be stronger than from questionnaire-based studies. The highest risk group had a 55-fold increased risk with no clear suggestion of a plateau in risk at high exposure levels, suggesting that analyzing the relationship between serum cotinine and lung cancer risk might contribute to a better quantitative assessment of tobacco-related lung carcinogenesis.[8] Additional prediagnostic urinary metabolites of tobacco carcinogens, such as NNAL (a metabolite of tobacco carcinogen NNK) and PheT (a metabolite of tobacco carcinogen polycyclic aromatic hydrocarbons [PAHs]), have also been associated with increased risk of lung cancer.[9,10] Other examples of biomarkers of internal dose include hormones or nutrients in body fluids and circulating antibodies to infectious agents (e.g., *Helicobacter pylori* in gastric cancer, hepatitis B virus [HBV] in liver cancer, and HPV in cervical cancer).[5]

Biomarkers of Biologically Effective Dose

Many carcinogens are metabolically activated and bind to DNA and/or protein to form adducts. DNA adducts have been the most evaluated biomarker of biologically effective dose, and a broad range of different DNA adducts have been measured in human samples using various approaches, including ^{32}P-postlabeling, immunoassays and immunohistochemistry, and mass spectrometry.[11,12] Because of the difficulty in measuring adducts in target tissues, almost all

of the population studies have used easily accessible surrogate samples, typically plasma/serum, white blood cells, or urine. The first prospective study showing a significant association between carcinogen-DNA adducts and subsequent cancer development was the aforementioned aflatoxin study in liver cancer.[6,13] Men with detectable urinary aflatoxin-DNA adduct (AFB1-N7-Guanine) and no HBV infection exhibited a ninefold increased liver cancer risk compared to men without detectable AFB1-N7-Guanine and no HBV infection. Tobacco smoke contains many different carcinogens, including PAHs, aromatic amines, and nitrosamines. Three prospective studies have evaluated the association of PAH-DNA or related aromatic-DNA adducts with the risk of lung cancer.[14-16] In a pioneering nested case-control study within the prospective Physicians' Health Study, the authors measured aromatic-DNA adducts in white blood cells (WBCs) at baseline and found current smokers who had higher levels of aromatic-DNA adducts in WBCs exhibited approximately threefold increased risk of lung cancer compared to current smokers with lower adduct concentrations. There were no associations in former and never smokers.[14] Two subsequent prospective studies, a pooled analysis of the three prospective studies, as well as a meta-analysis of nine studies (including retrospective case-control studies) recapitulated the main observation that bulky DNA adducts are associated with elevated lung cancer risk in current smokers after a follow-up of several years.[15-17] Protein adducts, such as hemoglobin (Hb) adducts of tobacco carcinogens and AFB1-albumin adducts, have also been used as biomarkers of biologically effective dose.[1,18,19]

Biomarkers of Effect

Biomarkers of effect measure early biological alterations that occur in the time frame between exposure and cancer development and are also known as biomarkers of intermediate endpoints or intermediate biomarkers. Historically, the most commonly studied biomarkers of effect in surrogate tissues are those related to genotoxicity endpoints, such as chromosome aberrations (CAs) and micronucleus (MN) formation in peripheral blood lymphocytes (PBLs). A number of prospective epidemiological studies have reported positive associations between elevated chromosomal aberrations in PBLs and increased cancer risk.[20-26] Importantly, a joint nested case-control study using subjects from two prospective cohort studies found that CA level in PBLs could predict future cancer development independent of environmental exposures (smoking and occupational exposure), supporting the notion that chromosomal instability markers could serve as both biomarkers of effect and biomarkers of susceptibility.[26] A few prospective studies showed that individuals with higher frequencies of MN in PBLs had 1.5- to 2-fold increased relative risk of cancer compared with the low-frequency subjects.[27,28]

Among various chromosome aberrations, chromosome translocation is one of the most well-established biomarkers of exposure and effect.[29] Translocations have been observed in nearly every cancer type,[30,31] and a single chromosome translocation can cause cancer—for example, Philadelphia translocation causes chronic myelogenous leukemia.[32,33] Chromosome translocation can survive mitosis and is the most persistent of all the different types of chromosome exchanges.[29] PBLs with radiation-induced translocations persisted for several decades in Japanese atomic bomb survivors. In contrast, other types of chromosome exchanges, for instance, dicentrics and acentric fragments, are unstable because they encounter difficulties during mitosis, which results in the affected cells being killed and eventually removed from the population, making them undetectable in long-term atomic bomb survivors.[34]

The mutational analyses of cancers with distinct environmental exposure provide elegant examples of biomarkers of effect. Exposure-specific mutational fingerprints in tumor suppressor genes have been known for many years.[35-39] The *TP53* gene is the most frequently mutated gene in human cancers, and its mutational spectrum varies substantially by tumor site, which is at least partially due to distinct environmental exposure.[36] For example, sunlight (UV exposure)-induced *TP53* mutations in skin cancer occur exclusively at dipyrimidine sites, including a high frequency of C-to-T transitions and unique CC-to-TT double base changes that do not happen in other malignancies.[37] In lung cancer, *TP53* mutational patterns are different between smokers and nonsmokers, with an excess of G-to-T transversions in smoking-associated cancers.[38] In liver cancer, a unique mutation (Arg249Ser) in *TP53* was linked to aflatoxin B1 exposure.[39,40] Moving beyond sequencing of candidate genes, the recent explosion of whole-genome sequencing (WGS) of cancer provides further evidence for profound effects of environmental exposure on the cancer genome.[41-44] In the first WGS of a solid tumor genome, Pleasance and colleagues[42] sequenced a malignant melanoma and a lymphoblastoid cell line from the same person. Consistent with observations in the *TP53* gene, of the more than 30,000 base substitutions found in the tumor genome compared to the lymphoblastoid genome, about 70% were C-to-T transitions. Of these, 92% occurred at the 3′ base of dipyrimidine sites, much higher than expected for chance occurrence. These mutations are characteristic of UVB-induced DNA lesions.[45] In the WGS of a small-cell lung cancer cell line[43] and a lung adenocarcinoma,[44] the mutational pattern (an excess of G-to-T mutations, an enrichment of CpG dinucleotides in the G-to-T

mutation sets, etc.) is also consistent with that of *TP53* and reflects the influences of tobacco carcinogens.[46]

With the breathtaking pace of technological advancements in the biomedical field, and with all the high-throughput "omics" technology, the list of potential biomarkers of effect is long and growing, encompassing molecular and functional changes in the epigenome, transcriptome, proteome, metabolome, and so on. There have been numerous investigations in exploring these technologies in epidemiological studies to identify biomarkers of effect in relation to cancer etiology; however, most of these studies are not validated, and the roles of the potential biomarkers in cancer causation are not clear. For biomarkers of effect (intermediate biomarkers), prospective studies are always preferred over retrospective case-control studies to avoid "reverse causation." Longitudinal evaluation of sequential samples should be the gold standard compared to cross-sectional and single time point analysis. There are many other issues in terms of study design, biospecimen collection and handling, assay reliability, and data reporting that are particularly important in the study of intermediate biomarkers to avoid spurious results.[3]

Biomarkers of Susceptibility

A major focus of cancer etiological study in recent years has been the determination of cancer susceptibility based on genetic variability. The earliest evidence for genetic susceptibility to cancer came from epidemiological observations of increased cancer risk among relatives of cancer patients. The existence of many rare inherited syndromes that predispose patients to increased risks of certain cancers provides other evidence for genetic susceptibility to cancer. A large classical twin study estimated the genetic heritability of most common cancers to be between 20% and 40%.[47] The identification of a large number of genetic susceptibility loci to common cancers by genome-wide association studies (GWAS) provides the strongest evidence for genetic susceptibility to common cancers.[48] Biomarkers of susceptibility have provided significant biological insight into cancer etiology and may become potential targets for preventive and therapeutic interventions. Biomarkers of susceptibility can also improve risk prediction and identify high-risk populations for targeted surveillance, screening, and prevention. Further investigation of gene-environment interactions is critical to advance the understanding of human carcinogenesis and improve the accuracy of cancer risk prediction.

Earlier analyses in cancer susceptibility have mostly focused on family-based linkage studies to identify high-penetrance genes whose mutations (mutation rate typically less than 0.1% in the general population) cause Mendelian cancer-predisposing syndromes. More recent efforts have mainly focused on association studies that compare variant allele frequencies of common single-nucleotide polymorphisms (SNPs) between a large number of cancer cases and unrelated controls. The underlying hypothesis for such an approach is the "common disease–common variant" (often abbreviated CD-CV) hypothesis, which postulates that genetic susceptibility to common diseases such as cancer is largely due to many common variants (typically having frequencies greater than 5% in the general population) with only modest effect conferred by each allele.[49] The competing hypothesis is the "common disease–rare variant" (CD-RV) hypothesis, which suggests that multiple rare variants (typically having frequencies that lie between approximately 0.1% and 1%, the upper limit for Mendelian mutations and the lower limit of SNPs, respectively) of larger effect cause common diseases.[50,51] However, recent evidence has suggested that these two hypotheses should not be viewed as an "either/or" choice, but rather that both common and rare variants may contribute to common diseases, and the degree of contribution by either variant depends on the particular disease phenotype.[50-52] In cancer, for example, and as described later, several rare susceptibility variants for breast cancer have been identified by candidate gene sequencing and genotyping. Furthermore, next-generation sequencing studies have identified novel rare susceptibility variants for other cancers.[53-57]

Depending on the population frequency of risk alleles and effect size, cancer genetic susceptibility markers can be roughly grouped into three classes: rare high-penetrance mutations, rare low- to moderate-penetrance disease-causing variants, and common low-penetrance SNPs (Table 21-1).[52]

Rare High-Penetrance Mutations

High-penetrance genes have been mostly identified by study of inherited Mendelian syndromes that predispose to cancer via linkage analysis followed by positional cloning. Such genes include *BRCA1* and *BRCA2* in hereditary breast/ovarian carcinoma; *RB1* in hereditary retinoblastoma; *APC* in familial adenomatous polyposis; mismatch repair genes, such as *MSH2*, *MLH1*, and *MSH6* in hereditary nonpoly posis colon carcinoma; and *VHL* in von Hippel–Lindau syndrome. Germline mutations in these high-penetrance genes confer high risk of respective cancers. However, because the population frequencies of these mutations are very rare (typically 0.1% or less), the population-attributable risk (PAR) is small.

Table 21-1 Three Classes of Biomarkers of Genetic Susceptibility to Cancer

	Rare High-Penetrance Mutations	Rare Low-to Moderate-Penetrance Variants	Common Low-Penetrance Variants
Population frequency	Rare, typically <0.1%	Rare, MAF typically between 0.1% and 2%	Common, MAF mostly >10%
Familial aggregation	Yes	No	No
Cancer risk (odds ratio)	≥10	Mostly ≥2	Mostly between 1.1 and 1.5
Population-attributable risk	Very small	Small	High
Functional significance	Direct effect (causal)	Direct effect (causal)	Mostly nonfunctional, in LD with causal variants
Approaches for identification	Linkage analysis followed by positional cloning, targeted sequencing of candidate genes	Candidate gene, exome, or whole genome sequencing of genetically enriched cases, followed by large-scale association study	Association study (candidate gene and GWAS) of unrelated cases and controls
Examples of genes	Breast cancer: BRCA1 and BRCA2; retinoblastoma: RB1; CRC: APC, mismatch repair genes (MSH2, MLH1, MSH6); kidney cancer: VHL	Breast cancer: ATM, CHEK2, BRIP1, PALB2, XRCC2; CRC: MUTYH; ovarian cancer: BRIP1; melanoma: MITF	Breast cancer: FGRF2, CASP8; lung cancer: CHRNA3-CHRNA5, TERT; CRC: TGFBR1, SMAD7, CDH1; bladder cancer: NAT2, GSTM1

CRC, Colorectal cancer; *GWAS*, genome-side association studies; *LD*, linkage disequilibrium; *MAF*, minor allele frequency.

Rare Low- to Moderate-Penetrance Variants

Rare low- to moderate-penetrance disease-causing variants are a relatively new territory in cancer susceptibility. However, the speed of identifying these variants is accelerating due to the ever-increasing application of next-generation sequencing (NGS) (whole exome or genome) in analysis of germline DNA. The earliest evidence for this class of variants came from rare variants in the *ATM* gene that increased breast cancer risk by two- to threefold,[58,59] whereas common SNPs in *ATM* were not associated with breast cancer.[60] Later on, rare variants in several *BRCA1-BRCA2* pathway genes, including *CHEK2*, *BRIP1*, and *PALB2*, were found to confer two- to threefold increased breast cancer risk.[61-63] In colorectal cancer (CRC), a large association study of 20,565 CRC cases and 15,524 controls uncovered that two rare variants, Gly396Asp (variant allele frequency, 0.7%) and Tyr179Cys (variant allele frequency, 0.2%), in the base excision repair gene *MUTYH* conferred extremely high risk of CRC: odds ratio (OR) = 23.1, 95% confidence interval [CI] 3.2 to 169; and OR = 4.1, 95% CI 0.9 to 18.5, respectively, for homozygous variant genotypes compared to the wild-type genotype.[64] Moreover, biallelic carriers of these two variants (i.e., homozygous at either site or heterozygous at both sites) exhibited a 28-fold (95% CI 6.9 to 115) increased CRC risk. This is currently the only example of a recessive model of inheritance for susceptibility to common cancers. Several recent studies have demonstrated the power of NGS in identifying novel rare cancer susceptibility variants.[53-55] In one study, Yokoyama and colleagues[53] first performed whole-exome sequencing (WES) of germline DNA from probands of several melanoma families and identified one novel variant (Glu318Lys) in the melanoma-lineage–specific oncogene *MITF*. Subsequently, they genotyped this rare variant in two large melanoma case-control studies conducted in the United States and Austria (a total of 3920 cases and 4036 controls) and found that in both studies carriers of the variant allele exhibited an over twofold increased risk of melanoma, with a combined OR of 2.19 (95% CI, 1.41 to 3.45). The variant allele frequency was 1.71% in cases compared to 0.79% in controls. Moreover, the variant allele was significantly overrepresented in cases with a family history of melanoma, multiple primary melanomas, or both. It was also associated with increased nevus count, non-blue eye color, and impaired *MITF* function in vitro, providing compelling evidence that *MITF* is a rare low-penetrance gene for melanoma. In another study, Park and co-workers[54] first performed WES of families with multiple cases of breast cancer and identified two families with *XRCC2* (a homologous recombination DNA repair gene) mutations. They then performed large-scale targeted sequencing of the *XRCC2* gene in a population-based case-control study (1308 cases with early-onset breast cancer and 1120 controls) that identified six probably pathogenic coding variants in cases but no variants in 1120 controls (*P* less than 0.02). Additional sequencing in 689 multiple-case families identified 10 families with protein-truncating or probably deleterious rare missense variants of *XRCC2*. Therefore, *XRCC2* is a novel rare low to moderately penetrant breast cancer susceptibility gene. A recent WES study of neuroblastoma found that rare, potentially pathogenic germline variants were significantly enriched in *ALK*, *CHEK2*, *PINK1*, and *BARD1*

genes.[57] WES-identified rare cancer susceptibility variants almost all have clear functional implications, and laboratory studies have been performed in many cases to confirm their functional effect. Thus, these markers are almost universally causal variants in causal genes.

WGS can provide enormous amounts of information (e.g., variants in regulatory regions such as noncoding RNAs) that are not available from WES. It can also open unprecedented opportunities in cancer association studies that will not only identify new cancer susceptibility loci but also facilitate the discovery of driver SNPs in regulatory regions. A recent study analyzed 32.5 million variants discovered by WGS of 1795 Icelanders and identified a new low-frequency variant at 8q24 that conferred a 2.9-fold increased risk of prostate cancer (PCa) ($P = 6.2 \times 10^{-34}$ in follow-up validation of more than 10,000 PCa cases and 62,000 controls of European origin).[65] Given the significantly reduced cost, WGS will be increasingly applied in cancer association studies.

Common Low-Penetrance SNPs

Accounting for 90% of naturally occurring genetic variations in the human genome, SNPs are the predominant marker of choice in current cancer association studies. By definition, SNPs have a minor allele frequency (MAF) of at least 1%. Most cancer association studies have examined common SNPs with an MAF of 5% or greater to allow for sufficient statistical power. Two approaches have been used to identify common SNPs as cancer susceptibility markers: the candidate gene approach and GWAS.

Candidate Gene Approach

The candidate gene approach stemmed from successful cellular phenotypic assays that were initiated before the development and application of genotyping techniques in epidemiological studies. Because of the important role of carcinogen metabolism and DNA repair pathways in determining cellular DNA adduct levels, it was natural to hypothesize that interindividual variations in these two cellular phenotypes may be associated with interindividual variation in exposure-induced cancer susceptibility. Therefore, cellular phenotypic assays in carcinogen metabolism and DNA repair capacity were initially used to determine interindividual variability in cancer susceptibility. Among such prototypic assays was evaluation of *N*-acetyltransferase (NAT) phenotypes based on NAT function as a phase II metabolizing enzyme involved in the detoxification of aromatic amines, which are known bladder carcinogens. The general population can be classified into rapid, intermediate, or slow acetylators, dependent on NAT activity, and an early phenotypic study suggested that slow acetylators were more susceptible to bladder cancer when exposed to bladder carcinogenic *N*-substituted aryl compounds.[66] Likewise, several different PBL-based DNA repair assays were widely applied in cancer association studies and yielded positive and consistent association between DNA repair capacity and cancer risk.[67] Starting from the early 1990s, with the development of low-throughput genotyping techniques, genetic association studies of cancer risk using a candidate gene approach exploded, initially focusing on carcinogen metabolism and DNA repair genes and later expanding to include other important cellular pathways. Numerous associations have been reported. However, most of these candidate gene studies involved small numbers of cases and controls, and very few of the initially reported positive susceptibility alleles have been replicated in subsequent validation studies.[68] Nevertheless, *NAT2* is among the very few validated cancer susceptibility loci derived from the candidate gene approach. As described earlier, NAT acetylator phenotypes are associated with bladder cancer risk, and these phenotypes can be tagged by genotypes composed of several SNPs in the *NAT2* gene. Numerous candidate gene studies and a meta-analysis have unequivocally confirmed that the *NAT2* slow acetylator genotype confers an approximately 50% increased risk of bladder cancer.[69] Other confirmed cancer susceptibility alleles from the candidate gene approach include the *GSTM1* null genotype in bladder cancer,[69] multiple SNPs of alcohol dehydrogenase genes (*ADH*) in upper aerodigestive cancers,[70] and a *CASP8* SNP (D302H) in breast cancer.[71] Besides carcinogen metabolism and DNA repair pathways, SNPs in other critical cellular pathways with obvious biological links to tumor development—for example, tumor microenvironment-related pathways such as immune surveillance and angiogenesis—have been extensively studied in relation to cancer susceptibility.[72-74] However, like most candidate gene studies, no SNP in these pathways has been unequivocally associated with cancer risks through a candidate gene approach.

Genome-Wide Association Studies

In the past 5 years, GWAS have revolutionized the study of cancer association and identified thousands of SNPs that are associated with the risk of many human diseases.[48] The GWAS approach tests hundreds of thousands of common SNP markers as surrogates to tag a much larger set of variants that are in strong linkage disequilibrium (LD) with the genotyped SNPs. Therefore, GWAS is discovery driven, in contrast to the hypothesis-driven candidate gene approach. The agnostic nature of GWAS allows identification of completely novel cancer susceptibility loci that would have been overlooked using the candidate gene approach. To date, more than 200 common susceptibility SNPs have been identified

for about two dozen cancer types.[48] These common susceptibility SNPs spread across all the chromosomes except the Y chromosome. A few regions, most notably 5p15.33 and 8q24, contain susceptibility SNPs for multiple cancer types (Figure 21-2). As expected from a tagging SNP-based approach, the vast majority of the identified SNPs are either intronic (39%) or intergenic (52%), and the functional implications of most SNPs are unclear.[75] Effect size associated with these common SNPs is modest (OR typically between 1.1 and 1.5).[75] Moreover, very few SNPs have an OR greater than 2, and the largest OR (over 2.5) has been observed for an SNP in the *KITLG* gene in testicular cancer, a malignancy with substantial familial risk but with little evidence for Mendelian predisposition.[76,77]

Most GWAS-identified SNPs are cancer-type specific and sometimes subtype specific. One example of a subtype-specific locus is in lung cancer, where 5p15.33 (*TERT-CLPTM1L* locus) is associated with adenocarcinoma but not squamous cell carcinoma.[78] However, there are at least seven regions that have been associated with more than one cancer type. For example, chromosome 8q24 flanking the *MYC* oncogene harbors susceptibility loci for prostate (five independent loci), breast, colon, bladder, and ovarian cancers as well as for chronic lymphocytic leukemia.[79] Although the *MYC* oncogene is the most likely plausible gene, these SNPs are relatively distant and are not in LD with any SNPs within the *MYC* gene or proximal promoter. Thus, just how these variants affect *MYC* function has been under extensive study. At least one CRC susceptibility SNP (rs6983267) in this region has been reported by two independent studies to be located within a transcriptional enhancer, bind differentially to transcription factor TCF7L2/TCF4, and either physically interact with the *MYC* gene[80] or enhance responsiveness of Wnt signaling, a key pathway in CRC.[81] Chromosome 5p15.33 (*TERT-CLPTM1L* locus) contains susceptibility loci for at least seven cancers, including melanoma, basal cell carcinoma, and bladder, brain, lung, pancreatic, and testicular cancer. In another example, a region on chromosome 11q13 harbors susceptibility SNPs for prostate, renal, and breast cancer.[79] In addition to these pleiotropic cancer susceptibility loci, a more interesting observation from GWAS is the pleiotropic susceptibility for seemingly unrelated genetic traits. However, at least some of these pleiotropic loci have biological plausibility. For example, an SNP in *TCF2* (a gene known to be involved in diabetes) was associated with a reduced risk of prostate cancer but an increased risk of type 2 diabetes (T2D),[82] consistent with previous epidemiological observation that individuals with T2D have a lower risk of developing prostate cancer.[83] An SNP in *ITPR2* was associated with an increased waist-hip ratio (WHR)[84] and increased risk of renal cell carcinoma (RCC).[85] Obesity is an established risk factor for RCC, and WHR has been suggested as a better measure of obesity than BMI. Other examples of pleiotropic loci in cancer and other genetic traits include the *JAZF1* locus with height,[86] T2D,[87] and prostate cancer[88] and the 9p21.3 (*CDKN2A/2B*) locus with T2D,[87] myocardial infarction,[89] nevus density,[90] melanoma,[91] and glioma.[92]

Identification of cancer-specific loci through GWAS has provided significant insight into the distinct genetic

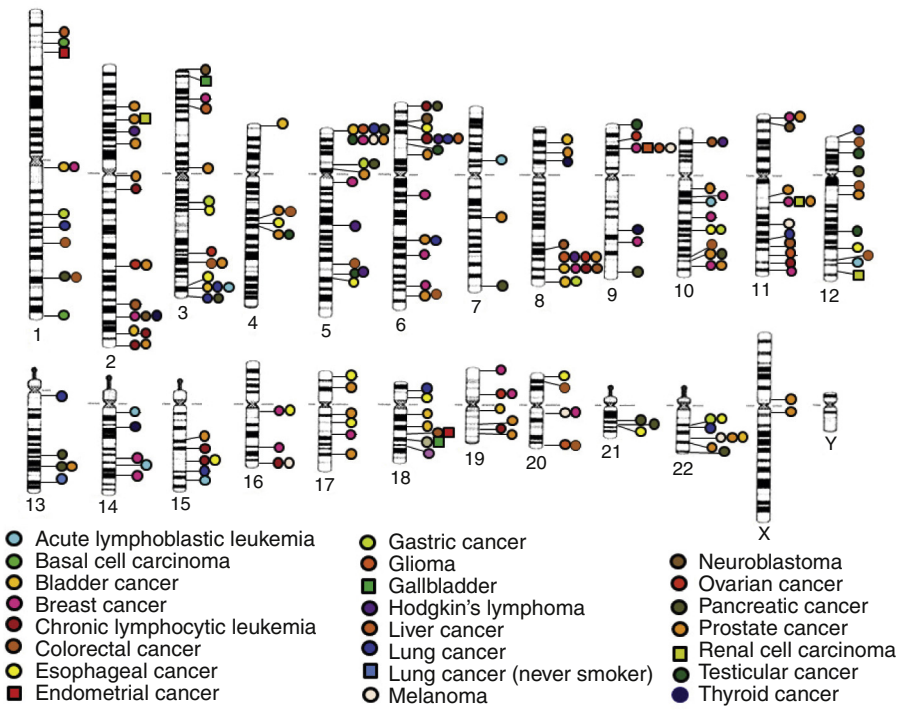


Figure 21-2 Genome-wide distribution of GWAS-identified cancer susceptibility loci Over 200 cancer susceptibility SNPs have been identified and these SNPs spread across all the chromosomes except the Y chromosome. The SNP data were obtained from the National Human Genome Research Institute (NHGRI) GWAS Catalog (http://www.genome.gov/gwastudies/).[48]

architecture of different cancers.[52,75] For example, PCa has the most susceptibility SNPs (over 70) identified to date, explaining about 30% of the familial risk in this disease.[93-95] Previously a classical twin study estimated a genetic heritability of 42% for PCa, the highest among common cancers[47]; however, a high-penetrance gene has yet to be identified and confirmed. One needs to reflect on underlying reasons for the high number of susceptibility SNPs discovered by GWAS in PCa. Whereas this could reflect the true genetic architecture of PCa wherein common variants with modest effect sizes play more prominent roles in PCa etiology, it is also possible that PCa may be more amenable to GWAS, possibly because of large sample size and limited confounding effects by environmental exposure and histological heterogeneity. In contrast to PCa, which does not have an obvious environmental risk factor, lung cancer is a strongly induced by environmental exposure. Perhaps reflecting the predominant effect of smoking, lung cancer is one of the malignancies with the least number of identified susceptibility SNPs. Multiple independent GWAS with very large sample sizes (13,300 cases and 19,666 controls in one study) have only unequivocally identified three susceptibility loci (15q25, 5p15, and 6p21) for lung cancer in individuals of European descent by stringent GWAS standards.[78,96-100] Importantly, the 15q25 variants were strongly associated with objective measures of tobacco exposure, suggesting that the association of these variants with lung cancer risk is likely to be mediated largely via tobacco exposure.[101] Bladder cancer lies in between PCa and lung cancer in terms of environmental exposure and genetic susceptibility and therefore is an excellent model for studying gene-environment interactions in cancer etiology. Smoking accounts for about half of all bladder cancers, and occupational exposure explains another one fifth. A large twin study estimated that inherited genetic susceptibility contributes to 31% of bladder cancer risk.[47] Eleven susceptibility loci have been validated for bladder cancer, and most of these loci have biological plausibility: three carcinogen-metabolizing genes, *GSTM1* on 1p13.3,[69,102] *NAT2* on 8p22,[69,103] and *UGT1A1* on 2q37.1[104]; a urea transporter gene *SLC14A1* on 18q12.3[105,106]; a cell cycle control gene *CCNE1* on 19q12[104]; and several oncogene, tumor-suppressor, or cell growth–related genes including *TP63* on 3q28,[107] *FGFR3* on 4p16.3,[108] *PSCA* on 8q24.3,[109] *TERT-CLPTM1L* on 5p15.33,[104,110] and a locus at 8q24.21, near *MYC*.[107] More importantly, there was a significant gene-smoking interaction between *NAT2* genotypes and smoking status, with the *NAT2* slow-acetylator genotype increasing bladder cancer risk, particularly among cigarette smokers.[69] This observation has become one of the classic examples of a gene-environment interaction in cancer etiology supported by a strong biological basis.

Based on the substantial number of cases and controls with both GWAS and exposure data, gene-environment interactions will be a hot topic in cancer etiology studies in the coming years. Indeed, a recent large-scale analysis of breast cancer highlighted the value of GWAS data together with exposure data.[111] A total of 34,793 invasive breast cancers and 41,099 controls of European ancestry from 24 studies of the Breast Cancer Association Consortium were analyzed for the modifying effect of 10 established environmental risk factors (age at menarche, parity, breastfeeding, body mass index, height, oral contraceptive use, menopausal hormone therapy use, alcohol consumption, cigarette smoking, and physical activity) on the association of 23 GWAS-identified SNPs with breast cancer risk. An SNP (rs3817198) in the *LSP1* gene showed strong interaction with parity (P for interaction = 2.4×10^{-6}): the per-allele OR (95% CI) was 1.08 (1.01 to 1.16) in nulliparous women and ranged from 1.03 (0.96 to 1.10) in parous women with one birth to 1.26 (1.16 to 1.37) in parous women with four or more births. Another SNP (rs17468277) in *CASP8* exhibited significant interaction with alcohol consumption (P for interaction = 3.1×10^{-4}): the per-allele OR (95% CI) was 0.91 (0.85 to 0.98) in those who drank less than 20 g/day and 1.45 (1.14 20 1.85) in those with an alcohol intake of at least 20 g/day.

Cellular Phenotypic Assays of Susceptibility

As introduced in earlier sections, cellular phenotypic assays in carcinogen metabolism and DNA repair capacity were initially used to determine interindividual variability in cancer susceptibility before the wide application of genotyping in cancer epidemiological studies. There is substantial interindividual variation in DNA repair capacity (DRC) in the human population. At the extreme end of this spectrum are patients with xeroderma pigmentosum, who have a defect in nucleotide excision repair and who exhibit a 1000-fold increased risk of skin cancer. There is a larger subgroup with suboptimal DRC who are phenotypically normal but may be at increased cancer risk if exposed to carcinogen. Various DNA repair assays in PBLs are still being used in assessing cancer risk. These assays include tests based on DNA damage induced by chemical or physical mutagen challenge, such as the mutagen sensitivity, induced micronuclei, and Comet assays; indirect tests of repair, such as unscheduled DNA synthesis; and direct measures of DNA repair capacity, such as the host cell reactivation assay.[67]

The mutagen sensitivity assay was the first DNA repair–related phenotypic assay to be widely applied in cancer epidemiological studies. Developed by Hsu and associates,[112] this assay cytogenetically quantifies the frequency of chromatid breaks induced by bleomycin in cultured PBLs as an indirect measure of DRC. The assay has since been expanded to employ other etiologically relevant mutagens

to assess genetic susceptibility to different cancers, such as benzo(a)pyrene [B(a)P] diolepoxide (BPDE) and NNK for smoking-related cancers, UV light for skin cancer, and γ-radiation for breast and brain cancer.[113] Numerous epidemiological studies, including a few prospective studies, have shown mutagen-sensitive phenotypes to be associated with increased risks of a variety of cancers.[113] Furthermore, classical twin studies have demonstrated that mutagen sensitivity is a genetic trait with heritability estimates ranging from 40% to 75%,[113,114] and a GWAS identified the first common SNP predictive of bleomycin-sensitive phenotype.[115] The data are compelling and support assay of mutagen sensitivity as a biomarker of cancer susceptibility.

The mutagen-challenged cytokinesis-blocked micronucleus (CBMN) assay in PBLs is another related and commonly used cytogenetic assay for DNA damage.[116] Micronuclei (MN) originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides. Because cells are blocked in the binucleated stage, it is also possible to measure nucleoplasmic bridges (NPBs) originating from asymmetrical chromosome rearrangements and/or telomere end fusions, and nuclear buds (NBUDs), the latter considered a marker of possible gene amplification. The CBMN assay has been used in assessment of cellular radiosensitivity to determine breast cancer predisposition and as a biomarker of susceptibility in tobacco-induced carcinogenesis.[116] A provocative retrospective case-control study showed that NNK-induced MN, NPBs, and NBUDs in PBLs were associated with a 2.3-, 45.5-, and 10.0-fold increased risk of lung cancer in smokers, respectively.[117] Whether such dramatic increases in risk hold up in prospective studies employing NNK-induced NPBs and NBUDs as biomarkers remains to be seen. Nevertheless, the simplicity, rapidity, and sensitivity of the CBMN assay make it a valuable tool for cancer risk assessment.

Unlike the cytogenetic assays that indirectly infer DNA repair, the host cell reactivation (HCR) assay is a direct measure of intrinsic DNA repair kinetics in intact cells. To perform this in vitro assay, PBLs are transfected with a damaged (treated by different mutagens) recombinant plasmid construct containing a reporter gene. The reporter gene's expression level directly reflects the PBLs' DRC to specific mutagens. The low DRC phenotype measured by HCR has been shown to be an independent risk factor for a variety of epithelial cancers in retrospective case-control studies.[118] Prospective studies are warranted to validate the HCR-measured DRC as valuable biomarkers of susceptibility.

In addition to DNA repair–related assays, telomere length in leukocytes, an indicator of biological aging and genomic instability, has been extensively evaluated as a cancer susceptibility marker in the past decade. The association appears to be cancer type–dependent. The majority of studies have shown significant associations between short telomeres in leukocytes and increased risks of cancers, including bladder, esophageal, gastric, head and neck, ovarian, and renal cancer; however, there were no significant associations reported in prostate, breast, and colorectal cancers from several large prospective studies.[119-122] Interestingly, two prospective studies reported that long telomeres were associated with increased risks of melanoma and non-Hodgkin lymphoma, respectively.[123,124] These cancer type–specific associations between telomere length and cancer risk warrant further validation and biological investigation.

Mouse Models for Cancer Susceptibility Study

Mouse models that cross tumor-resistant with tumor-susceptible strains have been instrumental in mapping several candidate cancer susceptibility loci and expression quantitative trait loci (eQTLs)[125-131] before the wide application of GWAS in human cancers. Although hundreds of cancer susceptibility loci have been identified through GWAS, the majority of the heritable risk of cancer cannot be explained by the main effects of common alleles. Gene-gene and gene-environment interaction clearly play important roles in cancer development, which is challenging in human studies because of the heterogeneity of human cancers. Mouse models have a defined genetic background that does not possess the genetic heterogeneity characteristic of human cancers. Crossing genetically distinct mouse strains can allow the analysis of the combinatorial effects of host genetic background and somatic events at different stages of cancer development. A recent study applied a network analysis in a mouse model of skin cancer that produces both benign tumors and malignant carcinomas and identified a genetic architecture affecting inflammation and tumor susceptibility.[132] Gene–environment interactions can also be investigated using mouse models to identify how genetic modifiers of tumor initiation interact with specific environmental effects identified through epidemiological studies. Mouse models will also be a major tool for mechanistic studies of cancer susceptibility loci.

Genetic Susceptibility to Cancer Outcomes

Genetic susceptibility plays a significant role not only in the initiation of cancer, but also in the outcomes of cancer treatment. Pharmacogenetics/pharmacogenomics is the study of the role of inherited and acquired genetic variation in drug response (efficacy and toxicity). Acquired somatic mutations and other types of abnormalities obviously play a determining role in patients' responses to therapeutic drugs—for instance, in the case of *HER2* overexpression in breast cancer and *EGFR* mutation in lung cancer. On the other hand, germline genetics may also modulate drug response, particularly systemic toxicities

associated with specific drugs. The most prominent example in this field is the effect of thiopurine *S*-methyltransferase (*TPMT*) gene variants on the toxicity of 6-mercaptopurine (6-MP), a drug used for the treatment of acute lymphoblastic leukemia (ALL). A standard dose of 6-MP would cause life-threatening toxicity to patients with certain variant alleles of *TPMT* that results in exceptionally low TPMT activity.[133,134] The U.S. Food and Drug Administration (FDA) has recommended *TPMT* genotyping and dosage reduction for those with low-activity alleles.[135] GWAS has also begun to be applied to pharmacogenomics research, although the sample size remains a major challenge. Ingle and colleagues presented an example of GWAS for outcomes.[136] Aromatase inhibitors have been used in estrogen-positive breast cancers to reduce recurrence. However, the administration of an aromatase inhibitor can also cause severe musculoskeletal pain in 10% to 20% of patients that may lead to therapy termination. Ingle and co-workers performed a small-scale GWAS using DNA samples from a large clinical trial of aromatase inhibitors in breast cancer patients and found a strong association—although not reaching genome-wide significance, likely because of small sample size—between musculoskeletal pain and variants in the gene cluster encoding T-cell leukemia lymphoma (TCL) proteins. Functional genomic studies showed that SNPs with the strongest association created an estrogen response element and that *TCL1A* expression was estrogen dependent and directly related to interleukin 17 receptor A (*IL17RA*) expression.[136] IL17RA is an experimental target for the treatment of patients with rheumatoid arthritis.[137] This provocative study illustrates the major challenge for GWAS of treatment responses: the difficulty of finding a large number of patients undergoing a specific drug treatment. Nevertheless, functional validation could increase the confidence that the observed genetic association (not reaching genome-wide significance) represents a true biological link rather than a chance finding. There have been increasing reports that applied GWAS to identify SNP association with the prognosis of common cancers[138-142]; however, there has not been a consistent susceptibility locus for any cancer outcome that is replicated by different groups. Future studies with large sample sizes and relatively homogeneous patient populations in terms of stage and treatment are warranted for GWAS of clinical outcomes. A detailed summary of cancer pharmacogenomics is outside the scope of this chapter, and readers are referred to other recent excellent review articles.[143,144]

Integrative Multifactor Risk Prediction

The ultimate goal of identifying biomarkers of cancer risk is to incorporate validated biomarkers into a comprehensive risk assessment model, with integration of environmental, behavioral, and genetic risk factors. The risk of cancer in the general population varies dramatically among different individuals, from average, to moderate, to high risk. For individuals with different cancer risks, there are different risk-reduction options. These include lifestyle changes for an average-risk person, and frequent screening and chemoprevention for moderate- and high-risk individuals. Cancer risk prediction models with high discriminatory accuracy can have significant applications in planning intervention trials, estimating the population burden of disease, creating benefit/risk indices and clinical decision-making processes, and designing prevention strategies. The currently developed cancer risk prediction models rely mostly on environmental risk factors and have modest discriminatory accuracy, which has limited clinical or public health utility. For example, heavy smoking confers an over 30-fold increased risk of lung cancer; however, consideration of smoking alone is insufficient to identify a high-risk population for cost-effective surveillance and screening. Indeed, the National Lung Screening Trial (NLST) showed that although low-dose computed tomography screening of heavy smokers can reduce lung cancer mortality by 20%, the feasibility of large-scale screening is limited by a false-positive rate of greater than 95%.[145,146] Improved risk prediction for lung cancer is critical to reduce false positives and shift the balance toward higher cost effectiveness for surveillance and screening. The best known cancer risk model, the Gail model for breast cancer, has a discriminatory accuracy of 0.58, measured as the area under the receiver operating characteristic curve (AUC), indicating that the distribution of projected risks among women who developed breast cancer overlapped considerably with that among women who did not develop breast cancer.[147] There have been many attempts to improve the modest discriminatory accuracy of the Gail model—for example, adding breast density in the BCRAT (*B*reast *C*ancer *R*isk *A*ssessment *T*ool of NCI, or Gail Model 2) modestly improved the discriminatory accuracy by 0.047.[148] Recent identification of multiple genetic predisposing loci for cancers by GWAS has generated great interest and raised hopes that these SNPs may improve risk prediction if incorporated into available risk-prediction models. However, the results have been somewhat disappointing. Addition of 7 and 11 GWAS-validated breast cancer susceptibility SNPs to the BCRAT model only improved the AUC by 0.025 and 0.030, respectively.[149,150] Also, adding three GWAS-validated SNPs to a lung cancer prediction model increased the AUC by 0.012.[151] As more and more common cancer susceptibility SNPs are identified through GWAS, there is clearly a potential to improve risk-prediction accuracy as more SNPs are incorporated. More importantly, gene–environment interactions have not been fully explored and integrated into

risk-prediction models, and this approach would probably add more discriminatory accuracy than integration of SNPs alone. Cellular phenotypic biomarkers (e.g., mutagen sensitivity and MN) are believed to measure the sum results of multiple genetic variations and thus, in theory, should provide more predictive power than individual SNPs. In the first attempt to incorporate cellular phenotypic biomarkers into a risk-prediction model, adding mutagen-sensitive phenotypes to a basic epidemiological risk prediction model for bladder cancer improved the AUC from 0.70 to 0.80.[152,153] This scale of increase is much higher than that reported from adding SNPs, demonstrating the potential of phenotypic biomarkers in improving cancer-risk prediction models.

Conclusion

Over the past two decades, enormous strides have been made in identifying biomarkers of cancer risk, and these discoveries have paralleled the evolution of the discipline of molecular epidemiology. Whereas the currently identified biomarkers have contributed greatly to our understanding of environmental exposure and cancer susceptibility, rapid advances in technology have greatly expanded the repertoire of biomarkers at all levels. We have already witnessed the revolution of GWAS in identifying novel cancer susceptibility factors. Although it is likely that most SNPs with larger ORs for common cancers have already been identified, given the large sample sizes of published studies, we will see continued application of GWAS in cancer risk assessment. Future GWAS will query cancer subtypes, population subgroups such as those categorized by exposure, and diverse populations. Exploring cancer susceptibility in diverse populations could further the understanding of disease pathways and assist in fine mapping of genetic associations by exploiting the differences in LD among different populations. Fine mapping and functional characterization of causal SNPs is a necessary step to move GWAS forward. GWAS will also be increasingly applied to identify genetic predictors of clinical outcomes. There is growing interest in the use of GWAS data for copy-number variation (CNV) analysis.[154] Moreover, the trend in performing WES and WGS to identify rare variants for cancer susceptibility will no doubt lead to an explosion of data reporting novel rare low- to moderate-penetrance variants, which would account for some of the missing heritability and provide enormous biological insight into human carcinogenesis. In the coming years, we will also see increasing use of various high-throughput "omics" technologies in cancer biomarker discovery. Unlike genotyping and sequencing, the techniques of epigenomics, transcriptomics, proteomics, and metabolomics are subjected to concerns of sample heterogeneity, technical reproducibility, and, if performed in retrospective case-control studies, "reverse causation." Longitudinal evaluation of these markers should always be preferred. Finally, validation is the key for any biomarkers, particularly those identified from high-throughput technologies. In conclusion, the overarching goal is to integrate different levels of biomarkers of cancer risk into comprehensive risk-prediction models for specific cancer types in order to benefit public health.

Key References

1. Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol Biomarkers Prev*. 2007;16:1954-1965.
2. Boffetta P. Biomarkers in cancer epidemiology: an integrative approach. *Carcinogenesis*. 2010;31:121-126.
3. Gallo V, Egger M, McCormack V, et al. STrengthening the Reporting of OBservational studies in Epidemiology—Molecular Epidemiology (STROBE-ME): an extension of the STROBE Statement. *PLoS Med*. 2011;8:e1001117.
4. Porta M. *A Dictionary of Epidemiology*. 5th ed. New York, NY: Oxford University Press; 2008 21.
5. Wild CP. Environmental exposure measurement in cancer epidemiology. *Mutagenesis*. 2009;24:117-125.
8. Boffetta P, Clark S, Shen M, et al. Serum cotinine level as predictor of lung cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2006;15:1184-1188.
9. Yuan JM, Gao YT, Murphy SE, et al. Urinary levels of cigarette smoke constituent metabolites are prospectively associated with lung cancer development in smokers. *Cancer Res*. 2011;71:6749-6757.
10. Yuan JM, Koh WP, Murphy SE, et al. Urinary levels of tobacco-specific nitrosamine metabolites in relation to lung cancer development in two prospective cohorts of cigarette smokers. *Cancer Res*. 2009;69:2990-2995.
11. Poirier MC. Chemical-induced DNA damage and human cancer risk. *Nat Rev Cancer*. 2004;4:630-637.
12. Rundle A. Carcinogen-DNA adducts as a biomarker for cancer risk. *Mutat Res*. 2006;600:23-36.
15. Peluso M, Munnia A, Hoek G, et al. DNA adducts and lung cancer risk: a prospective study. *Cancer Res*. 2005;65:8042-8048.
16. Bak H, Autrup H, Thomsen BL, et al. Bulky DNA adducts as risk indicator of lung cancer in a Danish case-cohort study. *Int J Cancer*. 2006;118:1618-1622.
17. Veglia F, Loft S, Matullo G, et al. DNA adducts and cancer risk in prospective studies: a pooled analysis and a meta-analysis. *Carcinogenesis*. 2008;29:932-936.
23. Hagmar L, Stromberg U, Bonassi S, et al. Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. *Cancer Res*. 2004;64:2258-2263.
24. Rossner P, Boffetta P, Ceppi M, et al. Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. *Environ Health Perspect*. 2005;113:517-520.
25. Boffetta P, van der Hel O, Norppa H, et al. Chromosomal aberrations and cancer risk: results of a cohort study from Central Europe. *Am J Epidemiol*. 2007;165:36-43.
27. Bonassi S, Znaor A, Ceppi M, et al. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*. 2007;28:625-631.

28. Murgia E, Ballardin M, Bonassi S, et al. Validation of micronuclei frequency in peripheral blood lymphocytes as early cancer risk biomarker in a nested case-control study. *Mutat Res*. 2008;639:27-34.
29. Tucker JD. Chromosome translocations and assessing human exposure to adverse environmental agents. *Environ Mol Mutagen*. 2010;51:815-824.
30. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. *Nat Rev Cancer*. 2007;7:233-245.
31. Frohling S, Dohner H. Chromosomal abnormalities in cancer. *N Engl J Med*. 2008;359:722-734.
33. Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. *Blood*. 2008;112:4808-4817.
39. Hussain SP, Schwank J, Staib F, et al. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. 2007;26:2166-2176.
40. Besaratinia A, Kim SI, Hainaut P, et al. In vitro recapitulating of TP53 mutagenesis in hepatocellular carcinoma associated with dietary aflatoxin B1 exposure. *Gastroenterology*. 2009;137:1127-1137 1137 e1–e5.
41. Pfeifer GP. Environmental exposures and mutational patterns of cancer genomes. *Genome Med*. 2010;2:54.
42. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010;463:191-196.
43. Pleasance ED, Stephens PJ, O'Meara S, et al. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature*. 2010;463:184-190.
44. Lee W, Jiang Z, Liu J, et al. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. *Nature*. 2010;465:473-477.
45. Pfeifer GP, You YH, Besaratinia A. Mutations induced by ultraviolet light. *Mutat Res*. 2005;571:19-31.
48. Hindorff L, MacArthur J (European Bioinformatics Institute), Wise A, Junkins HA, Hall PN, Klemm AK, Manolio TA. *A Catalog of Published Genome-Wide Association Studies*. Retrieved from www.genome.gov/gwastudies.
49. Lohmueller KE, Pearce CL, Pike M, et al. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet*. 2003;33:177-182.
50. Schork NJ, Murray SS, Frazer KA, et al. Common vs. rare allele hypotheses for complex diseases. *Curr Opin Genet Dev*. 2009;19:212-219.
51. Bodmer W, Bonilla C. Common and rare variants in multifactorial susceptibility to common diseases. *Nat Genet*. 2008;40:695-701.
52. Fletcher O, Houlston RS. Architecture of inherited susceptibility to common cancer. *Nat Rev Cancer*. 2010;10:353-361.
53. Yokoyama S, Woods SL, Boyle GM, et al. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. *Nature*. 2011;480:99-103.
54. Park DJ, Lesueur F, Nguyen-Dumont T, et al. Rare mutations in XRCC2 increase the risk of breast cancer. *Am J Hum Genet*. 2012;90:734-739.
55. Rafnar T, Gudbjartsson DF, Sulem P, et al. Mutations in BRIP1 confer high risk of ovarian cancer. *Nat Genet*. 2011;43:1104-1107.
56. Saarinen S, Aavikko M, Aittomaki K, et al. Exome sequencing reveals germline NPAT mutation as a candidate risk factor for Hodgkin lymphoma. *Blood*. 2011;118:493-498.
57. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45:279-284.
58. Thompson D, Duedal S, Kirner J, et al. Cancer risks and mortality in heterozygous ATM mutation carriers. *J Natl Cancer Inst*. 2005;97:813-822.
59. Renwick A, Thompson D, Seal S, et al. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat Genet*. 2006;38:873-875.
60. Baynes C, Healey CS, Pooley KA, et al. Common variants in the ATM, BRCA1, BRCA2, CHEK2 and TP53 cancer susceptibility genes are unlikely to increase breast cancer risk. *Breast Cancer Res*. 2007;9:R27.
62. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*. 2007;39:165-167.
63. Seal S, Thompson D, Renwick A, et al. Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet*. 2006;38:1239-1241.
65. Gudmundsson J, Sulem P, Gudbjartsson DF, et al. A study based on whole-genome sequencing yields a rare variant at 8q24 associated with prostate cancer. *Nat Genet*. 2012;44:1326-1329.
68. Dong LM, Potter JD, White E, et al. Genetic susceptibility to cancer: the role of polymorphisms in candidate genes. *JAMA*. 2008;299:2423-2436.
69. Garcia-Closas M, Malats N, Silverman D, et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. *Lancet*. 2005;366:649-659.
70. Hashibe M, McKay JD, Curado MP, et al. Multiple ADH genes are associated with upper aerodigestive cancers. *Nat Genet*. 2008;40:707-709.
71. Cox A, Dunning AM, Garcia-Closas M, et al. A common coding variant in CASP8 is associated with breast cancer risk. *Nat Genet*. 2007;39:352-358.
72. Kutikhin AG. Association of polymorphisms in TLR genes and in genes of the Toll-like receptor signaling pathway with cancer risk. *Hum Immunol*. 2011;72:1095-1116.
73. Spitz MR, Gorlov IP, Dong Q, et al. Multistage analysis of variants in the inflammation pathway and lung cancer risk in smokers. *Cancer Epidemiol Biomarkers Prev*. 2012;21:1213-1221.
74. Jain L, Vargo CA, Danesi R, et al. The role of vascular endothelial growth factor SNPs as predictive and prognostic markers for major solid tumors. *Mol Cancer Ther*. 2009;8:2496-2508.
75. Hindorff LA, Gillanders EM, Manolio TA. Genetic architecture of cancer and other complex diseases: lessons learned and future directions. *Carcinogenesis*. 2011;32:945-954.
76. Kanetsky PA, Mitra N, Vardhanabhuti S, et al. Common variation in KITLG and at 5q31.3 predisposes to testicular germ cell cancer. *Nat Genet*. 2009;41:811-815.
77. Rapley EA, Turnbull C, Al Olama AA, et al. A genome-wide association study of testicular germ cell tumor. *Nat Genet*. 2009;41:807-810.
78. Landi MT, Chatterjee N, Yu K, et al. A genome-wide association study of lung cancer identifies a region of chromosome 5p15 associated with risk for adenocarcinoma. *Am J Hum Genet*. 2009;85:679-691.
79. Chung CC, Chanock SJ. Current status of genome-wide association studies in cancer. *Hum Genet*. 2011;130:59-78.
80. Pomerantz MM, Ahmadiyeh N, Jia L, et al. The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. *Nat Genet*. 2009;41:882-884.
81. Tuupanen S, Turunen M, Lehtonen R, et al. The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat Genet*. 2009;41:885-890.
82. Gudmundsson J, Sulem P, Steinthorsdottir V, et al. Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. *Nat Genet*. 2007;39:977-983.
83. Kasper JS, Giovannucci E. A meta-analysis of diabetes mellitus and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2006;15:2056-2062.
84. Heid IM, Jackson AU, Randall JC, et al. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. *Nat Genet*. 2010;42:949-960.
85. Wu X, Scelo G, Purdue MP, et al. A genome-wide association study identifies a novel susceptibility locus for renal cell carcinoma on 12p11.23. *Hum Mol Genet*. 2012;21:456-462.
86. Soranzo N, Rivadeneira F, Chinappen-Horsley U, et al. Meta-analysis of genome-wide scans for human adult stature identifies novel loci and associations with measures of skeletal frame size. *PLoS Genet*. 2009;5:e1000445.
87. Voight BF, Scott LJ, Steinthorsdottir V, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet*. 2010;42:579-589.
88. Eeles RA, Kote-Jarai Z, Al Olama AA, et al. Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. *Nat Genet*. 2009;41:1116-1121.
89. Helgadottir A, Thorleifsson G, Manolescu A, et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science*. 2007;316:1491-1493.
90. Falchi M, Bataille V, Hayward NK, et al. Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. *Nat Genet*. 2009;41:915-919.
91. Bishop DT, Demenais F, Iles MM, et al. Genome-wide association study identifies three loci associated with melanoma risk. *Nat Genet*. 2009;41:920-925.
92. Shete S, Hosking FJ, Robertson LB, et al. Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet*. 2009;41:899-904.

93. Kote-Jarai Z, Olama AA, Giles GG, et al. Seven prostate cancer susceptibility loci identified by a multi-stage genome-wide association study. *Nat Genet*. 2011;43:785-791.
94. Nakagawa H, Akamatsu S, Takata R, et al. Prostate cancer genomics, biology, and risk assessment through genome-wide association studies. *Cancer Sci*. 2012;103:607-613.
95. Eeles RA, Olama AA, Benlloch S, et al. Identification of 23 new prostate cancer susceptibility loci using the iCOGS custom genotyping array. *Nat Genet*. 2013;45:385-391.
96. Amos CI, Wu X, Broderick P, et al. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet*. 2008;40:616-622.
97. Hung RJ, McKay JD, Gaborieau V, et al. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature*. 2008;452:633-637.
98. McKay JD, Hung RJ, Gaborieau V, et al. Lung cancer susceptibility locus at 5p15.33. *Nat Genet*. 2008;40:1404-1406.
99. Thorgeirsson TE, Geller F, Sulem P, et al. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature*. 2008;452:638-642.
100. Wang Y, Broderick P, Webb E, et al. Common 5p15.33 and 6p21.33 variants influence lung cancer risk. *Nat Genet*. 2008;40:1407-1409.
101. Munafo MR, Timofeeva MN, Morris RW, et al. Association between genetic variants on chromosome 15q25 locus and objective measures of tobacco exposure. *J Natl Cancer Inst*. 2012;104:740-748.
103. Gu J, Liang D, Wang Y, et al. Effects of N-acetyl transferase 1 and 2 polymorphisms on bladder cancer risk in Caucasians. *Mutat Res*. 2005;581:97-104.
104. Rothman N, Garcia-Closas M, Chatterjee N, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat Genet*. 2010;42:978-984.
105. Rafnar T, Vermeulen SH, Sulem P, et al. European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. *Hum Mol Genet*. 2011;20:4268-4281.
106. Garcia-Closas M, Ye Y, Rothman N, et al. A genome-wide association study of bladder cancer identifies a new susceptibility locus within SLC14A1, a urea transporter gene on chromosome 18q12.3. *Hum Mol Genet*. 2011;20:4282-4289.
107. Kiemeney LA, Thorlacius S, Sulem P, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. *Nat Genet*. 2008;40:1307-1312.
108. Kiemeney LA, Sulem P, Besenbacher S, et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. *Nat Genet*. 2010;42:415-419.
109. Wu X, Ye Y, Kiemeney LA, et al. Genetic variation in the prostate stem cell antigen gene PSCA confers susceptibility to urinary bladder cancer. *Nat Genet*. 2009;41:991-995.
110. Rafnar T, Sulem P, Stacey SN, et al. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet*. 2009;41:221-227.
111. Nickels S, Truong T, Hein R, et al. Evidence of gene-environment interactions between common breast cancer susceptibility loci and established environmental risk factors. *PLoS Genet*. 2013;9:e1003284.
113. Wu X, Gu J, Spitz MR. Mutagen sensitivity: a genetic predisposition factor for cancer. *Cancer Res*. 2007;67:3493-3495.
114. Wu X, Spitz MR, Amos CI, et al. Mutagen sensitivity has high heritability: evidence from a twin study. *Cancer Res*. 2006;66:5993-5996.
115. Gu J, Ye Y, Spitz MR, et al. A genetic variant near the PMAIP1/Noxa gene is associated with increased bleomycin sensitivity. *Hum Mol Genet*. 2011;20:820-826.
116. El-Zein R, Vral A, Etzel CJ. Cytokinesis-blocked micronucleus assay and cancer risk assessment. *Mutagenesis*. 2011;26:101-106.
117. El-Zein RA, Schabath MB, Etzel CJ, et al. Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Res*. 2006;66:6449-6456.
118. Li C, Wang LE, Wei Q. DNA repair phenotype and cancer susceptibility—a mini review. *Int J Cancer*. 2009;124:999-1007.
119. Wu X, Amos CI, Zhu Y, et al. Telomere dysfunction: a potential cancer predisposition factor. *J Natl Cancer Inst*. 2003;95:1211-1218.
120. Ma H, Zhou Z, Wei S, et al. Shortened telomere length is associated with increased risk of cancer: a meta-analysis. *PLoS One*. 2011;6:e20466.
121. Wentzensen IM, Mirabello L, Pfeiffer RM, et al. The association of telomere length and cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1238-1250.
122. Hou L, Zhang X, Gawron AJ, et al. Surrogate tissue telomere length and cancer risk: shorter or longer? *Cancer Lett*. 2012;319:130-135.
123. Han J, Qureshi AA, Prescott J, et al. A prospective study of telomere length and the risk of skin cancer. *J Invest Dermatol*. 2009;129:415-421.
124. Lan Q, Cawthon R, Shen M, et al. A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of non-Hodgkin lymphoma. *Clin Cancer Res*. 2009;15:7429-7433.
127. Ewart-Toland A, Briassouli P, de Koning JP, et al. Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nat Genet*. 2003;34:403-412.
128. Ewart-Toland A, Balmain A. The genetics of cancer susceptibility: from mouse to man. *Toxicol Pathol*. 2004;32(suppl 1):26-30.
129. Wakabayashi Y, Mao JH, Brown K, et al. Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. *Nature*. 2007;445:761-765.
130. Quigley D, Balmain A. Systems genetics analysis of cancer susceptibility: from mouse models to humans. *Nat Rev Genet*. 2009;10:651-657.
131. Quigley DA, To MD, Perez-Losada J, et al. Genetic architecture of mouse skin inflammation and tumour susceptibility. *Nature*. 2009;458:505-508.
132. Quigley DA, To MD, Kim IJ, et al. Network analysis of skin tumor progression identifies a rewired genetic architecture affecting inflammation and tumor susceptibility. *Genome Biol*.. 2011;12:R5.
135. Relling MV, Gardner EE, Sandborn WJ, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin Pharmacol Ther*. 2011;89:387-391.
136. Ingle JN, Schaid DJ, Goss PE, et al. Genome-wide associations and functional genomic studies of musculoskeletal adverse events in women receiving aromatase inhibitors. *J Clin Oncol*. 2010;28:4674-4682.
137. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med*. 2009;361:888-898.
138. Wu X, Ye Y, Rosell R, et al. Genome-wide association study of survival in non-small cell lung cancer patients receiving platinum-based chemotherapy. *J Natl Cancer Inst*. 2011;103:817-825.
139. Hu L, Wu C, Zhao X, et al. Genome-wide association study of prognosis in advanced non-small cell lung cancer patients receiving platinum-based chemotherapy. *Clin Cancer Res*. 2012;18:5507-5514.
140. Innocenti F, Owzar K, Cox NL, et al. A genome-wide association study of overall survival in pancreatic cancer patients treated with gemcitabine in CALGB 80303. *Clin Cancer Res*. 2012;18:577-584.
141. Kiyotani K, Mushiroda T, Tsunoda T, et al. A genome-wide association study identifies locus at 10q22 associated with clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients in Japanese. *Hum Mol Genet*. 2012;21:1665-1672.
142. Shu XO, Long J, Lu W, et al. Novel genetic markers of breast cancer survival identified by a genome-wide association study. *Cancer Res*. 2012;72:1182-1189.
143. Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. *N Engl J Med*. 2011;364:1144-1153.
144. Wheeler HE, Maitland ML, Dolan ME, et al. Cancer pharmacogenomics: strategies and challenges. *Nat Rev Genet*. 2013;14:23-34.
145. Bach PB, Mirkin JN, Oliver TK, et al. Benefits and harms of CT screening for lung cancer: a systematic review. *JAMA*. 2012;307:2418-2429.
146. Aberle DR, Adams AM, Berg CD, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med*. 2011;365:395-409.
148. Chen J, Pee D, Ayyagari R, et al. Projecting absolute invasive breast cancer risk in white women with a model that includes mammographic density. *J Natl Cancer Inst*. 2006;98:1215-1226.
149. Gail MH. Value of adding single-nucleotide polymorphism genotypes to a breast cancer risk model. *J Natl Cancer Inst*. 2009;101:959-963.
150. Gail MH. Discriminatory accuracy from single-nucleotide polymorphisms in models to predict breast cancer risk. *J Natl Cancer Inst*. 2008;100:1037-1041.

151. Spitz MR, Amos CI, D'Amelio Jr A, et al. Re: Discriminatory accuracy from single-nucleotide polymorphisms in models to predict breast cancer risk. *J Natl Cancer Inst*. 2009;101:1731-1732; author reply 1732.
152. Wu X, Lin J, Grossman HB, et al. Projecting individualized probabilities of developing bladder cancer in white individuals. *J Clin Oncol*. 2007;25:4974-4981.
153. Wu X, Hildebrandt MA, Chang DW. Genome-wide association studies of bladder cancer risk: a field synopsis of progress and potential applications. *Cancer Metastasis Rev*. 2009;28:269-280.
154. Jacobs KB, Yeager M, Zhou W, et al. Detectable clonal mosaicism and its relationship to aging and cancer. *Nat Genet*. 2012;44:651-658.

References

1. Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol Biomarkers Prev*. 2007;16:1954-1965.
2. Boffetta P. Biomarkers in cancer epidemiology: an integrative approach. *Carcinogenesis*. 2010;31:121-126.
3. Gallo V, Egger M, McCormack V, et al. STrengthening the Reporting of OBservational studies in Epidemiology—Molecular Epidemiology (STROBE-ME): an extension of the STROBE Statement. *PLoS Med*. 2011;8:e1001117.
4. Porta M. *A Dictionary of Epidemiology*. 5th ed. New York, NY: Oxford University Press; 2008 21.
5. Wild CP. Environmental exposure measurement in cancer epidemiology. *Mutagenesis*. 2009;24:117-125.
6. Ross RK, Yuan JM, Yu MC, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*. 1992;339:943-946.
7. Patrick DL, Cheadle A, Thompson DC, et al. The validity of self-reported smoking: a review and meta-analysis. *Am J Public Health*. 1994;84:1086-1093.
8. Boffetta P, Clark S, Shen M, et al. Serum cotinine level as predictor of lung cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2006;15:1184-1188.
9. Yuan JM, Gao YT, Murphy SE, et al. Urinary levels of cigarette smoke constituent metabolites are prospectively associated with lung cancer development in smokers. *Cancer Res*. 2011;71:6749-6757.
10. Yuan JM, Koh WP, Murphy SE, et al. Urinary levels of tobacco-specific nitrosamine metabolites in relation to lung cancer development in two prospective cohorts of cigarette smokers. *Cancer Res*. 2009;69:2990-2995.
11. Poirier MC. Chemical-induced DNA damage and human cancer risk. *Nat Rev Cancer*. 2004;4:630-637.
12. Rundle A. Carcinogen-DNA adducts as a biomarker for cancer risk. *Mutat Res*. 2006;600:23-36.
13. Qian GS, Ross RK, Yu MC, et al. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol Biomarkers Prev*. 1994;3:3-10.
14. Tang D, Phillips DH, Stampfer M, et al. Association between carcinogen-DNA adducts in white blood cells and lung cancer risk in the physicians health study. *Cancer Res*. 2001;61:6708-6712.
15. Peluso M, Munnia A, Hoek G, et al. DNA adducts and lung cancer risk: a prospective study. *Cancer Res*. 2005;65:8042-8048.
16. Bak H, Autrup H, Thomsen BL, et al. Bulky DNA adducts as risk indicator of lung cancer in a Danish case-cohort study. *Int J Cancer*. 2006;118:1618-1622.
17. Veglia F, Loft S, Matullo G, et al. DNA adducts and cancer risk in prospective studies: a pooled analysis and a meta-analysis. *Carcinogenesis*. 2008;29:932-936.
18. Chen CJ, Wang LY, Lu SN, et al. Elevated aflatoxin exposure and increased risk of hepatocellular carcinoma. *Hepatology*. 1996;24:38-42.
19. Wogan GN. Markers of exposure to carcinogens. *Environ Health Perspect*. 1989;81:9-17.
20. Hagmar L, Brogger A, Hansteen IL, et al. Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. *Cancer Res*. 1994;54:2919-2922.
21. Bonassi S, Abbondandolo A, Camurri L, et al. Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study. *Cancer Genet Cytogenet*. 1995;79:133-135.
22. Liou SH, Lung JC, Chen YH, et al. Increased chromosome-type chromosome aberration frequencies as biomarkers of cancer risk in a blackfoot endemic area. *Cancer Res*. 1999;59:1481-1484.
23. Hagmar L, Stromberg U, Bonassi S, et al. Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts. *Cancer Res*. 2004;64:2258-2263.
24. Rossner P, Boffetta P, Ceppi M, et al. Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer. *Environ Health Perspect*. 2005;113:517-520.
25. Boffetta P, van der Hel O, Norppa H, et al. Chromosomal aberrations and cancer risk: results of a cohort study from Central Europe. *Am J Epidemiol*. 2007;165:36-43.
26. Bonassi S, Hagmar L, Stromberg U, et al. Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health. *Cancer Res*. 2000;60:1619-1625.
27. Bonassi S, Znaor A, Ceppi M, et al. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*. 2007;28:625-631.
28. Murgia E, Ballardin M, Bonassi S, et al. Validation of micronuclei frequency in peripheral blood lymphocytes as early cancer risk biomarker in a nested case-control study. *Mutat Res*. 2008;639:27-34.
29. Tucker JD. Chromosome translocations and assessing human exposure to adverse environmental agents. *Environ Mol Mutagen*. 2010;51:815-824.
30. Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. *Nat Rev Cancer*. 2007;7:233-245.
31. Frohling S, Dohner H. Chromosomal abnormalities in cancer. *N Engl J Med*. 2008;359:722-734.
32. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243:290-293.
33. Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. *Blood*. 2008;112:4808-4817.
34. Awa A. Analysis of chromosome aberrations in atomic bomb survivors for dose assessment: studies at the Radiation Effects Research Foundation from 1968 to 1993. *Stem Cell*. 1997;15(suppl 2):163-173.
35. Hussain SP, Harris CC. Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res*. 1998;58:4023-4037.
36. Bennett WP, Hussain SP, Vahakangas KH, et al. Molecular epidemiology of human cancer risk: gene-environment interactions and p53 mutation spectrum in human lung cancer. *J Pathol*. 1999;187:8-18.
37. Brash DE, Rudolph JA, Simon JA, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 1991;88:10124-10128.
38. Pfeifer GP, Denissenko MF, Olivier M, et al. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene*. 2002;21:7435-7451.
39. Hussain SP, Schwank J, Staib F, et al. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. 2007;26:2166-2176.
40. Besaratinia A, Kim SI, Hainaut P, et al. In vitro recapitulating of TP53 mutagenesis in hepatocellular carcinoma associated with dietary aflatoxin B1 exposure. *Gastroenterology*. 2009;137:1127-1137 1137 e1–5.
41. Pfeifer GP. Environmental exposures and mutational patterns of cancer genomes. *Genome Med*. 2010;2:54.
42. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*. 2010;463:191-196.
43. Pleasance ED, Stephens PJ, O'Meara S, et al. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature*. 2010;463:184-190.
44. Lee W, Jiang Z, Liu J, et al. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. *Nature*. 2010;465:473-477.
45. Pfeifer GP, You YH, Besaratinia A. Mutations induced by ultraviolet light. *Mutat Res*. 2005;571:19-31.
46. Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst*. 1999;91:1194-1210.
47. Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med*. 2000;343:78-85.
48. Hindorff L, MacArthur J (European Bioinformatics Institute), Wise A, Junkins HA, Hall PN, Klemm AK, Manolio TA. *A Catalog of Published Genome-Wide Association Studies*. Retrieved from www.genome.gov/gwastudies.
49. Lohmueller KE, Pearce CL, Pike M, et al. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet*. 2003;33:177-182.
50. Schork NJ, Murray SS, Frazer KA, et al. Common vs. rare allele hypotheses for complex diseases. *Curr Opin Genet Dev*. 2009;19:212-219.
51. Bodmer W, Bonilla C. Common and rare variants in multifactorial susceptibility to common diseases. *Nat Genet*. 2008;40:695-701.
52. Fletcher O, Houlston RS. Architecture of inherited susceptibility to common cancer. *Nat Rev Cancer*. 2010;10:353-361.

53. Yokoyama S, Woods SL, Boyle GM, et al. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. *Nature*. 2011;480:99-103.
54. Park DJ, Lesueur F, Nguyen-Dumont T, et al. Rare mutations in XRCC2 increase the risk of breast cancer. *Am J Hum Genet*. 2012;90:734-739.
55. Rafnar T, Gudbjartsson DF, Sulem P, et al. Mutations in BRIP1 confer high risk of ovarian cancer. *Nat Genet*. 2011;43:1104-1107.
56. Saarinen S, Aavikko M, Aittomaki K, et al. Exome sequencing reveals germline NPAT mutation as a candidate risk factor for Hodgkin lymphoma. *Blood*. 2011;118:493-498.
57. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45:279-284.
58. Thompson D, Duedal S, Kirner J, et al. Cancer risks and mortality in heterozygous ATM mutation carriers. *J Natl Cancer Inst*. 2005;97:813-822.
59. Renwick A, Thompson D, Seal S, et al. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat Genet*. 2006;38:873-875.
60. Baynes C, Healey CS, Pooley KA, et al. Common variants in the ATM, BRCA1, BRCA2, CHEK2 and TP53 cancer susceptibility genes are unlikely to increase breast cancer risk. *Breast Cancer Res*. 2007;9:R27.
61. Meijers-Heijboer H, van den Ouweland A, Klijn J, et al. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet*. 2002;31:55-59.
62. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*. 2007;39:165-167.
63. Seal S, Thompson D, Renwick A, et al. Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat Genet*. 2006;38:1239-1241.
64. Laken SJ, Petersen GM, Gruber SB, et al. Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC. *Nat Genet*. 1997;17:79-83.
65. Gudmundsson J, Sulem P, Gudbjartsson DF, et al. A study based on whole-genome sequencing yields a rare variant at 8q24 associated with prostate cancer. *Nat Genet*. 2012;44:1326-1329.
66. Cartwright RA, Glashan RW, Rogers HJ, et al. Role of N-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer. *Lancet*. 1982;2:842-845.
67. Berwick M, Vineis P. Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. *J Natl Cancer Inst*. 2000;92:874-897.
68. Dong LM, Potter JD, White E, et al. Genetic susceptibility to cancer: the role of polymorphisms in candidate genes. *JAMA*. 2008;299:2423-2436.
69. Garcia-Closas M, Malats N, Silverman D, et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. *Lancet*. 2005;366:649-659.
70. Hashibe M, McKay JD, Curado MP, et al. Multiple ADH genes are associated with upper aerodigestive cancers. *Nat Genet*. 2008;40:707-709.
71. Cox A, Dunning AM, Garcia-Closas M, et al. A common coding variant in CASP8 is associated with breast cancer risk. *Nat Genet*. 2007;39:352-358.
72. Kutikhin AG. Association of polymorphisms in TLR genes and in genes of the Toll-like receptor signaling pathway with cancer risk. *Hum Immunol*. 2011;72:1095-1116.
73. Spitz MR, Gorlov IP, Dong Q, et al. Multistage analysis of variants in the inflammation pathway and lung cancer risk in smokers. *Cancer Epidemiol Biomarkers Prev*. 2012;21:1213-1221.
74. Jain L, Vargo CA, Danesi R, et al. The role of vascular endothelial growth factor SNPs as predictive and prognostic markers for major solid tumors. *Mol Cancer Ther*. 2009;8:2496-2508.
75. Hindorff LA, Gillanders EM, Manolio TA. Genetic architecture of cancer and other complex diseases: lessons learned and future directions. *Carcinogenesis*. 2011;32:945-954.
76. Kanetsky PA, Mitra N, Vardhanabhuti S, et al. Common variation in KITLG and at 5q31.3 predisposes to testicular germ cell cancer. *Nat Genet*. 2009;41:811-815.
77. Rapley EA, Turnbull C, Al Olama AA, et al. A genome-wide association study of testicular germ cell tumor. *Nat Genet*. 2009;41:807-810.
78. Landi MT, Chatterjee N, Yu K, et al. A genome-wide association study of lung cancer identifies a region of chromosome 5p15 associated with risk for adenocarcinoma. *Am J Hum Genet*. 2009;85:679-691.
79. Chung CC, Chanock SJ. Current status of genome-wide association studies in cancer. *Hum Genet*. 2011;130:59-78.
80. Pomerantz MM, Ahmadiyeh N, Jia L, et al. The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. *Nat Genet*. 2009;41:882-884.
81. Tuupanen S, Turunen M, Lehtonen R, et al. The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat Genet*. 2009;41:885-890.
82. Gudmundsson J, Sulem P, Steinthorsdottir V, et al. Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. *Nat Genet*. 2007;39:977-983.
83. Kasper JS, Giovannucci E. A meta-analysis of diabetes mellitus and the risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2006;15:2056-2062.
84. Heid IM, Jackson AU, Randall JC, et al. Meta-analysis identifies 13 new loci associated with waist-hip ratio and reveals sexual dimorphism in the genetic basis of fat distribution. *Nat Genet*. 2010;42:949-960.
85. Wu X, Scelo G, Purdue MP, et al. A genome-wide association study identifies a novel susceptibility locus for renal cell carcinoma on 12p11.23. *Hum Mol Genet*. 2012;21:456-462.
86. Soranzo N, Rivadeneira F, Chinappen-Horsley U, et al. Meta-analysis of genome-wide scans for human adult stature identifies novel loci and associations with measures of skeletal frame size. *PLoS Genet*. 2009;5:e1000445.
87. Voight BF, Scott LJ, Steinthorsdottir V, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet*. 2010;42:579-589.
88. Eeles RA, Kote-Jarai Z, Al Olama AA, et al. Identification of seven new prostate cancer susceptibility loci through a genome-wide association study. *Nat Genet*. 2009;41:1116-1121.
89. Helgadottir A, Thorleifsson G, Manolescu A, et al. A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science*. 2007;316:1491-1493.
90. Falchi M, Bataille V, Hayward NK, et al. Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. *Nat Genet*. 2009;41:915-919.
91. Bishop DT, Demenais F, Iles MM, et al. Genome-wide association study identifies three loci associated with melanoma risk. *Nat Genet*. 2009;41:920-925.
92. Shete S, Hosking FJ, Robertson LB, et al. Genome-wide association study identifies five susceptibility loci for glioma. *Nat Genet*. 2009;41:899-904.
93. Kote-Jarai Z, Olama AA, Giles GG, et al. Seven prostate cancer susceptibility loci identified by a multi-stage genome-wide association study. *Nat Genet*. 2011;43:785-791.
94. Nakagawa H, Akamatsu S, Takata R, et al. Prostate cancer genomics, biology, and risk assessment through genome-wide association studies. *Cancer Sci*. 2012;103:607-613.
95. Eeles RA, Olama AA, Benlloch S, et al. Identification of 23 new prostate cancer susceptibility loci using the iCOGS custom genotyping array. *Nat Genet*. 2013;45:385-391.
96. Amos CI, Wu X, Broderick P, et al. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet*. 2008;40:616-622.
97. Hung RJ, McKay JD, Gaborieau V, et al. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature*. 2008;452:633-637.
98. McKay JD, Hung RJ, Gaborieau V, et al. Lung cancer susceptibility locus at 5p15.33. *Nat Genet*. 2008;40:1404-1406.
99. Thorgeirsson TE, Geller F, Sulem P, et al. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature*. 2008;452:638-642.
100. Wang Y, Broderick P, Webb E, et al. Common 5p15.33 and 6p21.33 variants influence lung cancer risk. *Nat Genet*. 2008;40:1407-1409.
101. Munafo MR, Timofeeva MN, Morris RW, et al. Association between genetic variants on chromosome 15q25 locus and objective measures of tobacco exposure. *J Natl Cancer Inst*. 2012;104:740-748.
102. Bell DA, Taylor JA, Paulson DF, et al. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J Natl Cancer Inst*. 1993;85:1159-1164.
103. Gu J, Liang D, Wang Y, et al. Effects of N-acetyl transferase 1 and 2 polymorphisms on bladder cancer risk in Caucasians. *Mutat Res*. 2005;581:97-104.

104. Rothman N, Garcia-Closas M, Chatterjee N, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. *Nat Genet*. 2010;42:978-984.
105. Rafnar T, Vermeulen SH, Sulem P, et al. European genome-wide association study identifies SLC14A1 as a new urinary bladder cancer susceptibility gene. *Hum Mol Genet*. 2011;20:4268-4281.
106. Garcia-Closas M, Ye Y, Rothman N, et al. A genome-wide association study of bladder cancer identifies a new susceptibility locus within SLC14A1, a urea transporter gene on chromosome 18q12.3. *Hum Mol Genet*. 2011;20:4282-4289.
107. Kiemeney LA, Thorlacius S, Sulem P, et al. Sequence variant on 8q24 confers susceptibility to urinary bladder cancer. *Nat Genet*. 2008;40:1307-1312.
108. Kiemeney LA, Sulem P, Besenbacher S, et al. A sequence variant at 4p16.3 confers susceptibility to urinary bladder cancer. *Nat Genet*. 2010;42:415-419.
109. Wu X, Ye Y, Kiemeney LA, et al. Genetic variation in the prostate stem cell antigen gene PSCA confers susceptibility to urinary bladder cancer. *Nat Genet*. 2009;41:991-995.
110. Rafnar T, Sulem P, Stacey SN, et al. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet*. 2009;41:221-227.
111. Nickels S, Truong T, Hein R, et al. Evidence of gene-environment interactions between common breast cancer susceptibility loci and established environmental risk factors. *PLoS Genet*. 2013;9:e1003284.
112. Hsu TC, Johnston DA, Cherry LM, et al. Sensitivity to genotoxic effects of bleomycin in humans: possible relationship to environmental carcinogenesis. *Int J Cancer*. 1989;43:403-409.
113. Wu X, Gu J, Spitz MR. Mutagen sensitivity: a genetic predisposition factor for cancer. *Cancer Res*. 2007;67:3493-3495.
114. Wu X, Spitz MR, Amos CI, et al. Mutagen sensitivity has high heritability: evidence from a twin study. *Cancer Res*. 2006;66:5993-5996.
115. Gu J, Ye Y, Spitz MR, et al. A genetic variant near the PMAIP1/Noxa gene is associated with increased bleomycin sensitivity. *Hum Mol Genet*. 2011;20:820-826.
116. El-Zein R, Vral A, Etzel CJ. Cytokinesis-blocked micronucleus assay and cancer risk assessment. *Mutagenesis*. 2011;26:101-106.
117. El-Zein RA, Schabath MB, Etzel CJ, et al. Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Res*. 2006;66:6449-6456.
118. Li C, Wang LE, Wei Q. DNA repair phenotype and cancer susceptibility—a mini review. *Int J Cancer*. 2009;124:999-1007.
119. Wu X, Amos CI, Zhu Y, et al. Telomere dysfunction: a potential cancer predisposition factor. *J Natl Cancer Inst*. 2003;95:1211-1218.
120. Ma H, Zhou Z, Wei S, et al. Shortened telomere length is associated with increased risk of cancer: a meta-analysis. *PLoS One*. 2011;6:e20466.
121. Wentzensen IM, Mirabello L, Pfeiffer RM, et al. The association of telomere length and cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1238-1250.
122. Hou L, Zhang X, Gawron AJ, et al. Surrogate tissue telomere length and cancer risk: shorter or longer? *Cancer Lett*. 2012;319:130-135.
123. Han J, Qureshi AA, Prescott J, et al. A prospective study of telomere length and the risk of skin cancer. *J Invest Dermatol*. 2009;129:415-421.
124. Lan Q, Cawthon R, Shen M, et al. A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of non-Hodgkin lymphoma. *Clin Cancer Res*. 2009;15:7429-7433.
125. Nagase H, Bryson S, Cordell H, et al. Distinct genetic loci control development of benign and malignant skin tumours in mice. *Nat Genet*. 1995;10:424-429.
126. Ruivenkamp CA, van Wezel T, Zanon C, et al. Ptprj is a candidate for the mouse colon-cancer susceptibility locus Scc1 and is frequently deleted in human cancers. *Nat Genet*. 2002;31:295-300.
127. Ewart-Toland A, Briassouli P, de Koning JP, et al. Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nat Genet*. 2003;34:403-412.
128. Ewart-Toland A, Balmain A. The genetics of cancer susceptibility: from mouse to man. *Toxicol Pathol*. 2004;32(suppl 1):26-30.
129. Wakabayashi Y, Mao JH, Brown K, et al. Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. *Nature*. 2007;445:761-765.
130. Quigley D, Balmain A. Systems genetics analysis of cancer susceptibility: from mouse models to humans. *Nat Rev Genet*. 2009;10:651-657.
131. Quigley DA, To MD, Perez-Losada J, et al. Genetic architecture of mouse skin inflammation and tumour susceptibility. *Nature*. 2009;458:505-508.
132. Quigley DA, To MD, Kim IJ, et al. Network analysis of skin tumor progression identifies a rewired genetic architecture affecting inflammation and tumor susceptibility. *Genome Biol*. 2011;12:R5.
133. Weinshilboum RM, Sladek SL. Mercaptopurine pharmacogenetics: monogenic inheritance of erythrocyte thiopurine methyltransferase activity. *Am J Hum Genet*. 1980;32:651-662.
134. Relling MV, Hancock ML, Rivera GK, et al. Mercaptopurine therapy intolerance and heterozygosity at the thiopurine S-methyltransferase gene locus. *J Natl Cancer Inst*. 1999;91:2001-2008.
135. Relling MV, Gardner EE, Sandborn WJ, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin Pharmacol Ther*. 2011;89:387-391.
136. Ingle JN, Schaid DJ, Goss PE, et al. Genome-wide associations and functional genomic studies of musculoskeletal adverse events in women receiving aromatase inhibitors. *J Clin Oncol*. 2010;28:4674-4682.
137. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med*. 2009;361:888-898.
138. Wu X, Ye Y, Rosell R, et al. Genome-wide association study of survival in non-small cell lung cancer patients receiving platinum-based chemotherapy. *J Natl Cancer Inst*. 2011;103:817-825.
139. Hu L, Wu C, Zhao X, et al. Genome-wide association study of prognosis in advanced non-small cell lung cancer patients receiving platinum-based chemotherapy. *Clin Cancer Res*. 2012;18:5507-5514.
140. Innocenti F, Owzar K, Cox NL, et al. A genome-wide association study of overall survival in pancreatic cancer patients treated with gemcitabine in CALGB 80303. *Clin Cancer Res*. 2012;18:577-584.
141. Kiyotani K, Mushiroda T, Tsunoda T, et al. A genome-wide association study identifies locus at 10q22 associated with clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients in Japanese. *Hum Mol Genet*. 2012;21:1665-1672.
142. Shu XO, Long J, Lu W, et al. Novel genetic markers of breast cancer survival identified by a genome-wide association study. *Cancer Res*. 2012;72:1182-1189.
143. Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. *N Engl J Med*. 2011;364:1144-1153.
144. Wheeler HE, Maitland ML, Dolan ME, et al. Cancer pharmacogenomics: strategies and challenges. *Nat Rev Genet*. 2013;14:23-34.
145. Bach PB, Mirkin JN, Oliver TK, et al. Benefits and harms of CT screening for lung cancer: a systematic review. *JAMA*. 2012;307:2418-2429.
146. Aberle DR, Adams AM, Berg CD, et al. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med*. 2011;365:395-409.
147. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst*. 1989;81:1879-1886.
148. Chen J, Pee D, Ayyagari R, et al. Projecting absolute invasive breast cancer risk in white women with a model that includes mammographic density. *J Natl Cancer Inst*. 2006;98:1215-1226.
149. Gail MH. Value of adding single-nucleotide polymorphism genotypes to a breast cancer risk model. *J Natl Cancer Inst*. 2009;101:959-963.
150. Gail MH. Discriminatory accuracy from single-nucleotide polymorphisms in models to predict breast cancer risk. *J Natl Cancer Inst*. 2008;100:1037-1041.
151. Spitz MR, Amos CI, D'Amelio Jr A, et al. Re: Discriminatory accuracy from single-nucleotide polymorphisms in models to predict breast cancer risk. *J Natl Cancer Inst*. 2009;101:1731-1732; author reply 1732.
152. Wu X, Lin J, Grossman HB, et al. Projecting individualized probabilities of developing bladder cancer in white individuals. *J Clin Oncol*. 2007;25:4974-4981.
153. Wu X, Hildebrandt MA, Chang DW. Genome-wide association studies of bladder cancer risk: a field synopsis of progress and potential applications. *Cancer Metastasis Rev*. 2009;28:269-280.
154. Jacobs KB, Yeager M, Zhou W, et al. Detectable clonal mosaicism and its relationship to aging and cancer. *Nat Genet*. 2012;44:651-658.

Susan Fisher and H. Ewa Witkowska

22

Protein Biomarkers for Detecting Cancer: Molecular Screening

No matter how many mistakes you make or how slow you progress, you are still way ahead of everyone who isn't trying.

(http://www.marcandangel.com/2012/06/08/60-quotes-change-the-way-you-think/)

Biomarkers of early cancer detection, specific markers of a malignancy type, and predictive markers of response to treatment will aid in the early diagnosis and selection of the most efficient therapies. An exponential growth in technologies has been achieved toward this goal in the past decade. However, it is safe to say that the field of disease biomarkers produced many more publications on the subject than actual "clinically actionable" targets. By no means a criticism, this statement reflects on the status quo of a discipline that has been "trying really hard" but finding the goal more elusive with each step forward.[1] Why has the progress been slow? Is it just the technology that is not on par with the complexity of human biology? Or perhaps, by focusing mainly on a paradigm of "DNA-mRNA-protein" as a fundamental driving force that defines a phenotype, are we oversimplifying and hence misinterpreting a system?[2,3] We bring these very general questions to the attention of the reader for two reasons: first to spark a debate on the fundamental issues that biomarker discovery entails and second to put our detailed proteomics discussion into the perspective within the larger context of biomarker discovery.

Establishing a panel of biomarkers for early diagnosis of cancer holds tremendous promise but also faces daunting obstacles. The major challenge stems from the very nature of a biological system, its complexity, dynamics, variability, and versatility all making it difficult to draw clear lines between a state that would be considered normal and one that appears to be slightly different and in danger of becoming abnormal. Then come barriers of logistics, such as a dichotomy between a need to mainly rely on population studies while facing tremendous intra- and interpersonal phenotypic variability, defining adequate controls, availability of specimens, and the danger of their potential adulteration before analysis (i.e., in the course of collection, processing, and storage). Last but not least is the requirement for sensitive technologies capable of measuring target compounds directly from a biological milieu with a level of specificity and selectivity that allows differentiation among discrete cohorts of individuals/patients that is reliable enough to justify the risk of acting on specific clinical modalities. There is a growing recognition that achieving true breakthroughs in the use of biomarkers to improve human conditions, while delivering health care in an economically sustainable way, requires concerted and coordinated efforts of stakeholders across disciplines and across borders. A large consortium of authors has recently published an in-depth analysis of the status of implementation of proteomics biomarkers of disease with a focus on postdiscovery/postvalidation barriers and outlined a need for a roadmap to biomarker implementation.[4] In our review, we concentrate on the preimplementation stages of the biomarker pipeline from the perspective of technologies that are currently available for biomarker discovery and validation, with a focus on protein biomarkers for which mass spectrometry (MS) plays a primary role.

The discipline of MS-based proteomics[5] emerged through a serendipitous convergence of major technological developments in DNA sequencing, MS analysis of proteins/peptides and bioinformatics. In the enthusiasm that followed the success of the Human Genome Project, it appeared that understanding how the functional phenotype of a cell/tissue/organism relates to its protein/peptide repertoire was imminent, leading to exaggerated expectations that the new technology of proteomics would deliver meaningful results in a short time. A rush for quick success resulted in controversies and disappointments, and unavoidably triggered questions as to the fundamental validity and practical usefulness of proteomics approaches.[6-8] The involvement of funding agencies (e.g., the National Cancer Institute [NCI]), which funded programs aimed at addressing various stumbling blocks of

proteomics technologies (e.g., the Clinical Proteomics Technologies for Cancer),[9-15] and of professional organizations and consortia (e.g., the Human Proteome Organization [HUPO][16-20] and the Proteomics Specification in Time and Space [PROSPECTS] Network[21]) has been crucial, providing funding and building foundations and collaborations among various disciplines to ensure future success in developing protein-based biomarkers of disease. Now, after more than a decade of intense effort, the promise of proteomics remains valid. Accumulated experience, however, required reassessment of the prospects of achieving quick payoffs—especially in the context of translational research, as illustrated by a recent breast cancer biomarker study using an animal model that is reviewed at the end of this chapter. Nevertheless, the field is progressing steadily, and the lessons learned in the early days of the proteomics bonanza are informing the directions of new developments.[22-30] Thousands of papers on the subject have been published to date. Here we focus on selected aspects of method development that, in our opinion, will play a significant role in moving this effort toward its eventual success.

Before discussing different aspects of technology for protein biomarker discovery, it is important to ponder the nature of the objects of inquiry, that is, a protein and a proteome, in the context of the goal of finding biomarkers of physiological processes leading to tumorigenesis. At first approximation, a protein can be viewed as a gene that has been realized in a dimension defined by the available amino acid building blocks. With this simple though simplistic concept of a protein-gene equivalence in mind, the proteome can be reduced to a catalogue of gene products present in a cell. From this perspective, experiments aimed at monitoring gross changes in the repertoire of "protein parts"—that is, the presence or absence of a specific gene product, or significant differences in protein levels—would lead to the detection of robust biomarkers. Most protein biomarker discovery studies to date were based on these operational definitions of a protein and a proteome. The adopted strategies represented a natural extension of familiar approaches used in functional genomics and took advantage of the availability of the required, albeit imperfect, experimental tools. However, although proteins start their lives as strings of amino acid residues arranged in an order prescribed by DNA, they further acquire a multitude of attributes that endow them with functional competence: they fold into specific shapes; they might need to be "cut to order" by proteolytic enzymes; they are decorated with modifications, many of which are transient by design; they are localized to the proper compartment of a cell; they interact with other cell components, including other proteins, to form "molecular machines"; and, once their mission has been fulfilled, they need to be disposed of in an orderly fashion. Defects in any of these processes could lead to, or result from, disease and hence could be used as valuable biomarkers.[31-36] However, because of the very nature of experimental design and confounding factors of system complexity and limitations of technology, these types of biomarker candidates are likely to be missed in studies geared solely toward detecting changes in peptide/protein concentration. Although technically more challenging, approaches that target specific attributes of protein structure and function, including but not limited to the examples listed earlier, are gaining importance in the biomarker discovery field. Of note, systems biology–based predictions are still far from being able to model all aspects of protein "life." Thus the analysis of a final protein product is the only approach currently available for characterization of protein-driven cell processes. As we discuss in this chapter, MS-based proteomics approaches enable exploration of biomarkers in various contexts.

Defining "Normal"

"Always remember that you are absolutely unique. Just like everyone else." This maxim, attributed to Margaret Mead, is also an excellent comment on the challenges intrinsic to biomarker discovery. What is normal for one person might be outside the norm for another. Hence, the "perfect world" scenario of early detection of malignancy, or any other malady, would use "self" as a control and rely on serial analyses of specimens collected at different time points, thus focusing on changes in, rather than absolute levels of, putative markers of health or disease. For a variety of reasons, longitudinal collection of specimens for the population at large is not feasible. Therefore, we are limited to epidemiological approaches. In this context, assessing the level of variability within a population is vital to provide a baseline/reference point for evaluating disease consequences in terms of biomarkers.[37] To this end, a number of tools have been proposed and/or are being developed. A protein equivalent to the HapMap,[38] and repositories of human DNA sequences,[39-41] is the foundation on which disease-related changes in protein repertoire will need to be built. Databases of MS-identified peptides are being generated for human[42] and model organisms (e.g., mouse[43]). In addition, the normal range of post-translational modifications (PTMs) should be considered.[44] For example, population proteomics proposes using targeted affinity capture approaches combined with high-throughput MS to screen large numbers of samples for a broad array of modifications.[45] Antibodies or lectins could be used for enrichment. In this regard, the Human Protein Atlas is a valuable resource with respect to antibodies,[46,47] and it will be important to generate the lectin equivalent.[48,49] Examples of these datasets include publications on the normal urine,[50]

breast,[51] oral epithelium,[52] liver,[53,54] and brain[55] proteomes and PTMs in various settings.[56] These catalogues of normal proteomes and PTMs will be crucial for identifying disease-related changes—that is, candidate biomarkers.

MS Proteomics for Biomarker Discovery and Validation: An Overview of Basic Methods

The ideal biomarker discovery methods should be comprehensive to sift through as many potential targets as possible; verification/validation methods should be specific and accurate to filter out all false positives. The "open-mindedness" of MS in detecting all species that are ionizable, transferable to the gas phase, and produce discernible signals under the chosen experimental conditions, makes it an excellent biomarker discovery tool (Figure 22-1). Because it is not necessary to know beforehand the identities of compounds to be monitored, untargeted (shotgun) approaches can generate extensive information on sample content.[57-59] Conversely, MS assays can also be executed in a targeted fashion by focusing data acquisition on prespecified compounds of interest,[60] thus providing the high level of selectivity required for biomarker validation experiments. In their most sophisticated format, verification/validation assays employ standards labeled with stable isotopes to enable high-sensitivity

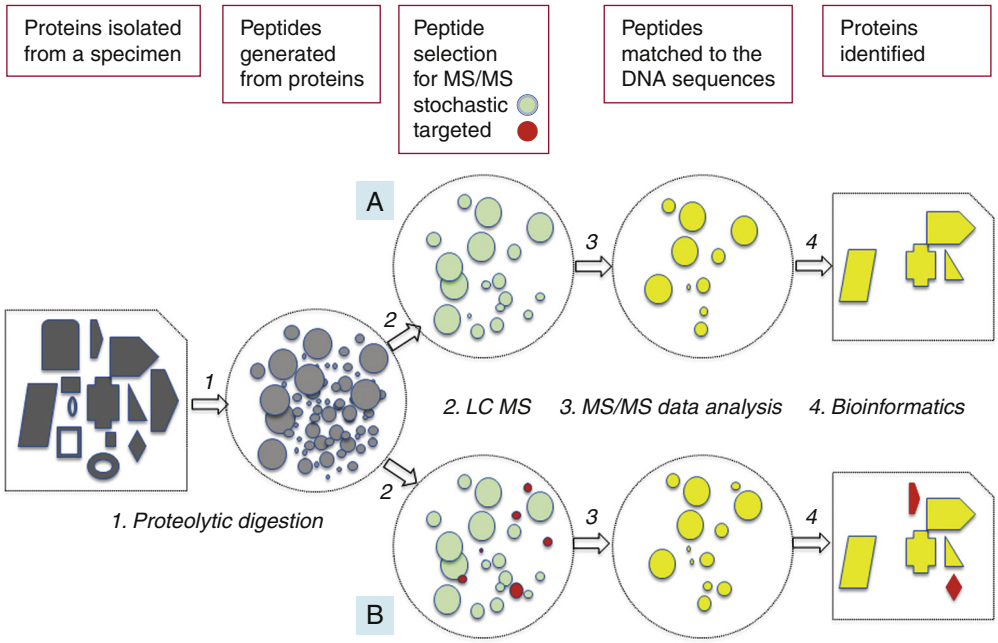


Figure 22-1 Shotgun **(A)** and hybrid **(B)** proteomics discovery workflows. A protein mixture isolated from a specimen consists of a great variety of species present at different concentrations (the size of a "protein shape" reflects its relative abundance). In either workflow, only a fraction of proteins/peptides that are present in the sample are identified. At step 1, proteins are cut to smaller, manageable pieces using proteolytic enzyme(s), most commonly trypsin, to generate peptides (*circles;* different sizes represent relative concentrations). The resulting peptide mixtures, which typically contain hundreds of thousands of species, are prefractionated using chromatography (not shown) and then submitted to LC MS analysis (step 2). To identify a peptide, and hence the protein from which it was derived, peptide ions must be fragmented into smaller, sequence-dependent parts. Peptide fragmentation is performed within the mass spectrometer and is referred to as *tandem mass spectrometry* (MS/MS). Most of the MS/MS approaches currently used rely on analyzing each peptide molecular ion (precursor) one by one. Given the complexity of a sample, at any given time the mass spectrometer is presented with many more precursors than it can possibly analyze by MS/MS. Hence, ion prioritization for further MS/MS analysis is necessary. In an untargeted, shotgun workflow **(A)**, selection of a peptide molecular ion for fragmentation is based on signal intensity, leading to a stochastic choice of precursors from within a large array of potential contenders. Predictably, shotgun analysis is biased toward selecting highly abundant peptides at the expense of less abundant ones. In the hybrid workflow **(B)**, a predefined set of precursor ions of interest is selected for MS/MS (step 1) followed by a shotgun signal intensity-based acquisition routine (step 2), as described for **(A)**. The targeted ions are analyzed by MS/MS regardless of their relative abundance, as long as they meet the analysis-wide signal-to-noise threshold for precursor ion intensity (note the small size of the *red* dots representing target peptides selected for MS/MS in workflow B). In step 3, the experimental MS/MS spectra are compared to the theoretical in situ generated mock spectra predicted for peptides representing all proteins for which DNA sequences are known. In this process, the peptide identities and hence proteins are not derived de novo from the MS/MS data. Rather, they represent the best matches between the observed and theoretically predicted spectra. Of note, typically some of the molecular ions selected for MS/MS analysis do not generate reliable matches, and hence the number of identified peptides is always lower than the number of acquired MS/MS spectra. In step 4, bioinformatics tools are used to combine peptide-based matches to generate a list of proteins that are most likely present in the mixture. Because of the targeted nature of workflow **B**, low-abundance proteins *(red)* that are missed by the shotgun workflow **(A)** can now be identified. Thus, using a targeted approach for discovery overcomes the limitations of stochastic ion selection. Hence, to compare the compositions of two samples it is necessary to target the species of interest in both samples rather than rely on the unbiased nature of the stochastic shotgun workflow.

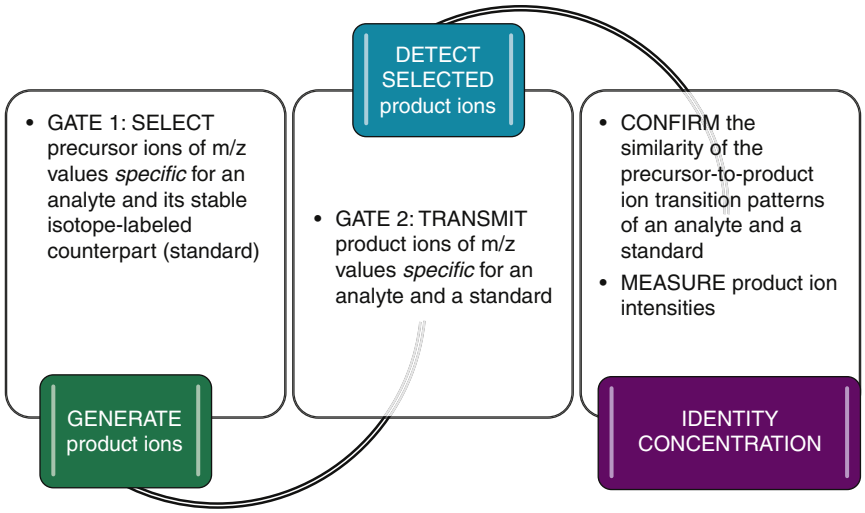


Figure 22-2 Outline of stable isotope dilution multiple reaction monitoring mass spectrometry (SID MRM MS) experiments SID MRM MS workflows provide highly reliable and sensitive detection and accurate measurement of absolute concentrations of endogenous analytes such as proteins. The assay is reliable because double-gated filtering is used at both the precursor-ion (gate 1) and the product-ion (gate 2) stages of analysis. This method is very sensitive because the mass spectrometer exclusively monitors ions of interest without interference from others present in the sample. Analyte concentrations are measured with high accuracy by using stable-isotope (SI)-labeled internal standards that have identical chemistry to the endogenous analytes. Typically, SI-labeled internal standards are spiked into the sample at known levels early on in the experimental workflow to account for losses encountered during sample processing. The ability to measure absolute concentrations of analytes provides a high level of confidence when comparing compositions of specimens obtained from different patient cohorts in biomarker verification and validation studies.

detection, structure confirmation, and absolute quantification of selected protein targets (Figure 22-2). In contrast to classical immunochemistry-based approaches, MS-based biomarker validation assays—that is, *stable isotope dilution (SID) multiple reaction monitoring (MRM)* MS (also called *selected reaction monitoring* [SRM] MS)—do not rely on protein-specific antibodies, and offer high multiplexing capabilities.[9,61-64] To achieve ultimate sensitivity in detecting and quantifying selected proteins, immunoaffinity isolation of representative target peptides followed by MRM is used and is referred to as *stable isotope standards with capture by anti-peptide antibodies* (SISCAPA).[65] Last, recent developments in MS technology—that is, improvements in mass resolution leading to significantly higher accuracy in detecting true targets[66,67]—and greatly enhanced scan rates, enabled hybrid MS discovery workflows in which the targeted and untargeted methods are executed in a single analysis. These hybrid workflows allow for the preferential analysis of predetermined ions while the excess capacity is used to analyze "unknowns" (see Figure 22-1, *B*).

Discovery and verification/validation platforms share a number of analytical steps albeit their modes of execution can differ significantly. Typically, liquid chromatography (LC) is used to fractionate a sample before MS analysis. Eluting compounds are either transferred online in a continuous fashion to the mass spectrometer for electrospray ionization (ESI) MS[68] or collected offline in discrete fractions for subsequent matrix-assisted laser desorption ionization (MALDI) MS.[69] The classical experimental approach to proteomics MS data acquisition, which still dominates the field, involves rapidly toggling between two distinct automatically executed modes of operation: MS and tandem MS (MS/MS). The MS mode operates under conditions that maintain the molecular integrity of analytes and delivers a survey of mass-to-charge ratios (m/z) and relative intensities of the detected molecular ions (precursors). During MS/MS, a subset of precursor ions are transferred, one by one, to a collision cell where they are dissociated under controlled conditions into a series of fragment (product) ions whose m/z values and relative intensities carry structure-specific information. Although the elemental steps are common for both verification/validation and discovery platforms, the paradigms of data acquisition and analysis differ significantly. In the former case, precursor ion selection before MS/MS is centered on predetermined masses of the components of interest; all other species in the sample are ignored. In the latter case, the targets are yet to be discovered and, hence, precursors are selected in a stochastic fashion. As to data analysis, in discovery assays, the experimental MS/MS spectra are compared to the theoretical in situ generated mock spectra predicted for peptides representing all proteins for which DNA sequences are known. In verification/validation assays (SID MRM MS), confirmation of analyte identity is based on matching the MS/MS data to the previously experimentally established, rather than predicted, MS/MS fragmentation features (i.e., types and relative intensities of product ions).

Completeness of Shotgun Biomarker Discovery Proteomics

Limitations Inherent to Peptide-Centric Strategies for Protein Biomarker Discovery

With the exception of protein profiling, described later, the great majority of workflows that are currently used for protein biomarker discovery and validation rely on the analysis of peptides generated from proteins via enzymatic or chemical digestion. Consequently, the information about the structure of a protein identified in the sample is limited to a portion of amino acid sequence that is encompassed by the observed peptides (Figure 22-3). When the goal of analysis is to detect differences in relative protein concentration, limited sequence coverage by detected peptides often leads to ambiguity in identifying highly homologous proteins. Most importantly, any subtle but functionally relevant disease-related structural modifications resulting from posttranscriptional/posttranslational processing are likely to remain invisible when shotgun approaches are used (see Figure 22-3). To remedy this problem, a number of specialized methods based on either chemical or biochemical characteristics of specific modifications (e.g., affinity capture using antibodies or lectins) are being developed to increase the chances of detecting biomarkers that reflect proteome alterations extending beyond changes in protein expression and/or degradation. The ideal solution would be to use a protein-centric approach: to analyze intact proteins by MS to directly reveal potential changes in highly heterogeneous protein populations. Presently, high-resolution MS and MS/MS analysis of intact proteins in biological samples remains technically challenging. However, great strides in instrument design, along with novel method development, continue to advance this area of proteomics research.[70-72]

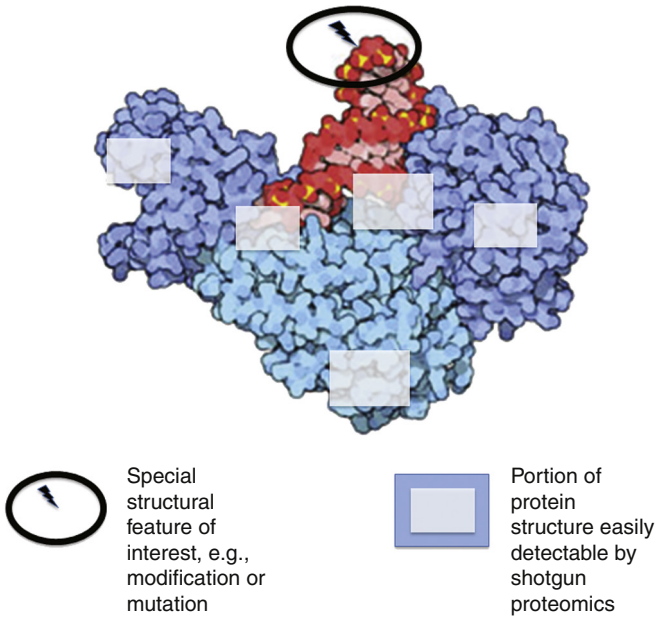


Figure 22-3 Challenges and opportunities presented by special features of protein structure, e.g., protein posttranslational modifications in proteomics analysis There is no guarantee that an untargeted shotgun analysis will detect the portion of the protein structure that carries a modification (*oval;* parts of protein sequence covered by identified peptides are shown by *gray rectangles*). Importantly, even if a peptide carrying a modification is seen, it might not be identified, that is, matched to a DNA sequence by using currently available software algorithms. Hence, if detection of a specific modification is a primary goal, specialized methods that target specific chemical/biochemical features of the modification need to be used. For example, affinity capture of glycoproteins using lectins or capture of phosphotyrosine residues using antibodies can be performed. Use of affinity capture or other specialized methods allows enrichment of subsets of proteins/peptides carrying specific modifications, thus enabling identification of protein scaffolds present at very low levels. *The figure template is based on David Goodsell's rendering of Aconitase and Iron Regulatory Protein 1 (May 2007 Molecule of the Month) by David Goodsell, retrieved from the RCSB Protein Data Bank at* http://www.rcsb.org/pdb/pdb/101/motm.do?momID=42*; doi: 10.2210/rcsb_pdb/mom_2007_5.*

Technical Limitations of Data Generation

The great complexity and large dynamic range of protein concentrations in biological samples present major challenges to all MS proteomics workflows because of a mismatch between the number of molecular ions generated and the MS analyzer's capacity to process them by MS/MS. As a result, a large fraction of the detectable ions are not identified in a single MS/MS experiment (see Figure 22-1). Furthermore, because of the combination of the inherent nonlinearity of the system, and stochastic selection of precursors, subsets of ions analyzed by MS/MS will not be identical across replicate experiments, generating dissimilar, albeit overlapping, protein/peptide lists.[15] Adverse effects of crowding-related competition lead to undersampling,[73] affecting untargeted discovery platforms to a much higher degree than targeted approaches. The actual extent of these limitations was demonstrated through a series of controlled, parallel benchmarking experiments, which established that prior knowledge of signature peptides (i.e., peptides having sequenced that are unique to targeted proteins) markedly improves overall detection sensitivity and reliability of quantification.[74] Performing replicate LC MS runs increases the number of identified m/z features. To maximize efficiency and information content, it is advantageous to set up replicate analyses in an intelligent fashion to ensure that the same set of molecular ions will be interrogated across all samples, while allowing room for new discoveries in the course of each iteration. To this end, the hybrid untargeted/targeted workflows described earlier are best suited to monitor specific molecular ions that were missed in parallel analyses, thus minimizing information gaps in sample sets.[75,76] Undersampling tends to skew the results in favor of the most abundant

species, especially when ion intensity is used as the sole criterion for precursor selection—that is, when "data-dependent" routines are used. Thus, novel iterative "information-dependent" data acquisition routines are being developed that intelligently prioritize targets for tandem MS on the basis of previously collected data.[77-81]

Limitations in Data Analysis

Currently, protein identification is based on matching the experimentally acquired peptide MS/MS spectra to theoretical in silico predictions derived from DNA sequences, rather than on inferring the sequence information by de novo interpretation of the observed signature fragment ions.[82] Hence, the success of analysis—assignment of peptide MS/MS spectra to specific proteins—relies to a great extent on the selection and quality of the protein sequence database that is being interrogated. Important parameters include relevance to the experimental design and the completeness and accuracy of the underlying DNA sequencing data. The need for the selection of a representative database cannot be overstated. First, proteins that are not included in the database will not be identified. Second, when there is a mismatch between sample composition and the database that is searched, there exists a serious danger of generating false-positive identifications. The example of identifying iridovirus and the microsporidian *Nosema* as purported reasons for bee colony collapse offers an important cautionary tale.[83] The authors performed proteomics analysis of material isolated from bees, but restricted the database to sequences of potential pathogenic organisms while excluding those of the host. Independent analyses of the data clearly demonstrated that MS/MS spectra originally assigned to iridovirus and *Nosema* were actually matched to the bee protein sequences with much higher probability.[84]

MS Protein Profiling

In addition to the high-resolution MS-based methods described earlier, *protein profiling* based on low-resolution MS analysis of mixtures of intact proteins in body fluids has been extensively used in biomarker discovery research.[85] In its most common format of surface enhanced laser desorption/ionization (SELDI) MS,[86,87] proteins from body fluids such as serum or plasma are affinity captured by surfaces with various chemical properties and, following the addition of a matrix, ionized under MALDI conditions and surveyed using a time-of-flight (TOF) mass analyzer. The resulting mass spectra show a series of *m/z* features (peaks) of various intensities. The premise of this method is that the SELDI pattern will reliably discriminate among samples of various biological fluids/cells/tissues that are collected in normal versus disease states without invoking the need for identifying the diagnostic features. Intrinsic to this approach is the concept of developing biomarker panels rather than single protein sentinels, which is appealing given the multifactorial nature of neoplastic diseases. Although attractive because of the simplicity of concept and methods, and its high-throughput potential, SELDI MS protein profiling is plagued by a number of problems. First, the identities of proteins on which the diagnostic pattern is based are not known. Second, in view of the limitations of mass resolution, it is impossible to tell whether each peak represents a single protein or a mixture of species of similar molecular masses. A combination of these two factors makes it difficult to control method reproducibility. Third, even small changes in sample composition greatly affect the efficiencies of molecular ion generation under MALDI conditions. Hence, altered relative intensities of the same peak in different samples might be due to secondary effects rather than a reflection of differences in concentration.[88] Last, SELDI is unlikely to capture low-abundance proteins. For example, many of the discriminatory proteins reported for ovarian cancer, such as apolipoprotein A, haptoglobin, and complement component 3, were also found to be affected in a number of different types of cancers. Nevertheless, the first proteomics-derived protein test for cancer biomarkers that was approved by the U.S. Food and Drug Administration (FDA) originated from SELDI technology, although its final format is based on a standard immunoassay, rather than an MS platform. The OVA1 test for ovarian cancer diagnosis (21 CFR 866.6050, ovarian adnexal mass assessment score test system) was developed by Vermillion, Inc.,[89] approved by the FDA in 2009, and launched commercially by Quest Diagnostics in 2010. This assay quantifies five serum proteins: beta-2-microglobulin, prealbumin, apolipoprotein A-1, transferrin, and CA125. The results are scored from 1 to 10 to classify the likelihood that a woman's pelvic mass is cancerous or benign.[90] Of note, a recent SELDI-based study that monitored seven serum proteins (some of them included in the OVA1 test) in addition to CA125 in prediagnostic specimens did not demonstrate any improvements in sensitivity for preclinical diagnosis of ovarian cancer when compared to using CA125 as the only factor.[91] In a recent paper evaluating SELDI technology, the authors reviewed 34 SELDI studies focused on ovarian cancer and compared the published data to their own analysis of plasma from ovarian cancer patients using MALDI techniques.[92] They found nearly a 50% overlap among all the detected *m/z* features and a 34% overlap in a subset of discriminating peaks, concluding that the results "indicate convergence toward a set of common discriminating, reproducible peaks for (identifying) ovarian cancer." Hence, although the SELDI

or MALDI protein profiling platforms are not adequate for biomarker validation and clinical diagnosis, they might still have a niche at the discovery stage of biomarker development, particularly when they are used in combination with MALDI methods of identification. However, the serious limitations of SELDI technology discussed earlier should be fully taken into consideration before choosing this path.

The Proteomics Toolbox for Biomarker Discovery and Validation

Developments in Mass Spectrometry Instrumentation and Acquisition Methods

As previously noted, undersampling is one of the bottlenecks in achieving increased breadth of proteome interrogation. In addition to the information-dependent modes of precursor ion selection that were discussed earlier, a number of other ideas for maximizing the generation of structural information from detected peptides are being pursued. An obvious way to increase the number of precursor ions that can be analyzed on the time scale of their introduction to the mass spectrometer is to significantly increase the speed of MS/MS acquisition. MS vendors are moving quickly in this direction. A paradigm shift in thinking about MS/MS experiments brought about revolutionary approaches that eliminate precursor ion selection altogether (parallel acquisition[93]). In these strategies, such as MS^{E}[94,95] and SWATH,[96,97] molecular ions introduced into the mass spectrometer are subjected to gas-phase fragmentations en masse in the source.[95-97] Software tools are used to disentangle the resulting spectra, linking product ions to their precursors, and thus assigning peptide identities to precursor ions. This brute-force approach has become feasible because of the high accuracy of product ion mass measurements afforded by the current instrumentation and the ability to capitalize on shared product and precursor ion elution profiles to facilitate the analysis.[93,94]

Another way of overcoming the crowding problem is to minimize the load of precursors that need to be processed by the mass spectrometer within a unit of time. Sample simplification via increased fractionation is being extensively used, with myriad variations that might include protein separation before protein digestion and/or extensive separation of peptide mixtures via a combination of orthogonal LC or capillary electrophoresis methods.[98] Other improvements in increasing the efficiency of MS protein identification are likely to come from the recently commercialized technology of ion mobility separation (IMS).[99-101] IMS fractionates ions in the gas phase on the basis of the ratio of their collision cross-section to their charge—that is, "shape"-to-charge ratios—thus providing an additional dimension of molecular ion separation *after* sample introduction into the mass spectrometer. Mass spectrometers equipped with IMS analyzers are capable of separating species of the same nominal mass that have different higher-order structures, thus increasing the visibility of ions that might have otherwise been missed and hence not considered for MS/MS analysis. Last but not least, samples can be simplified by removing interfering species and/or enriching for proteins/peptides of interest, as discussed later.

Targeted MS Assays for Biomarker Discovery and Qualification for Analysis of Splice Variants

Alternative splicing has long been considered a hallmark of malignant transformation,[102-105] and recent progress in RNA-Seq technologies revealed thousands of aberrant transcripts in tumor tissues. However, in the great majority of cases, nothing is known about the functional implications of these alterations. Indeed, only a small fraction of potential splice variants have been observed at the protein level. The task of unambiguous identification of a splice variant is not trivial.[106] It requires the ability to differentiate the aberrant protein from other variant and normal isoforms that are likely to be present in the same sample. Whereas proving the presence of a gene product requires identification of any peptide from any part of a protein sequence, the diagnostic field of opportunity for a splice variant detection often is restricted to a very small portion of the protein sequence that encompasses the splice junction. Peptides diagnostic for splice forms stand the same chance of being selected for MS/MS in untargeted workflows as any other peptides of similar properties and abundance, as evidenced by a number of successful studies.[104,107-109] However, we posit that use of targeted workflows might prove much more productive in detecting specific splice variants. It is important to note that not every splice junction will be represented by a tryptic peptide that generates a precursor that can be analyzed by MS. Hence, use of various proteolytic strategies and complementary MS ionization modes—that is, MALDI and ESI—will likely increase the number of identified splice variants.

General Affinity-Based Methods to Access Low-Abundance Proteins

Body fluids, especially blood, are considered the primary source of cancer biomarkers because of their diagnostic availability and high likelihood that they contain a cancer fingerprint that represents proteins secreted, shed, or otherwise deposited into circulation from distant tumor locations. At the same time, plasma and serum present with formidable

analytical challenges due to their complexity in terms of the number of proteins that they carry, as well as the dynamic range of those proteins' concentrations.[110] Specifically, out of thousands of proteins likely present in plasma/serum, fewer than 20 account for more than 95% of the protein mass. Despite the currently achievable mid-attomole level of peptide detection, the dynamic range of plasma proteins, exceeding 10 orders of magnitude, is yet to be matched by the analytical capabilities of the most advanced mass spectrometers. Hence, strategies involving the depletion of the most abundant sample components have been widely adopted, mainly based on immunoaffinity approaches using IgG and IgY antibodies.[111-116] Although such a strategy enables much deeper interrogation of the proteome than would be otherwise feasible, it comes with a price. Namely, potential biomarkers that strongly bind to proteins targeted for removal will also be depleted and hence lost forever from further analyses.[113,117] The full extent of this potential problem is not presently known, but efforts toward evaluating the "omics" of a depleted fraction, currently focused mainly on proteins/peptides bound to albumin—the albuminome—are being pursued.[118-120] Of note, these studies could turn a problem into a solution: Abundant proteins targeted for depletion could be used as affinity reagents for enriching a specific subset of interacting proteins. In this regard, the "depletome" is a relatively untapped source of putative biomarkers.[121]

The very different approach of using intermolecular interactions to access the low-abundance end of the proteome is based on the concept of "equalizing" protein concentrations within the sample.[122,123] Rather than removing abundant species, each protein, regardless of its relative concentration, is given a statistically even chance of encountering a suitable epitope to which it would bind with high affinity. Importantly, all possible epitopes are delivered at equimolar concentrations that are significantly lower than the concentrations of high- and medium-abundance proteins. In this scenario, it is the number of high-affinity epitopes that are available rather than the overall amount of a protein in the original sample that would drive final composition of the extracted protein mixture. This approach was commercialized as ProteoMiner technology by BioRad and uses a library of hexapeptides attached to magnetic beads. Although the concept of dynamic range compression offered by equalizer beads is very appealing, it is yet to be optimized. The bulky physical format might be one of the limiting factors; a large volume of beads is required to achieve a comprehensive representation of all potential epitopes, thus necessitating the use of matching volumes of a sample. Nevertheless, the method was successfully applied to profile more than 1500 low-abundance proteins in erythrocyte lysates, a sample presenting great challenges because hemoglobin (Hb) comprises 98% of the cells' proteome. However, to achieve this feat, 5 g of total protein was used to isolate 8 mg of material—an estimated recovery of about 8% of the non-Hb fraction.[124] In plasma, antibody depletion and peptide ligand library enrichment methods performed similarly.[125] In addition, the utility of the latter capture method as applied to the analysis of conditioned medium, which contained serum, was recently shown.[126] A number of studies have been published that used the peptide ligand library approach in the context of cancer biomarker discovery.[127-129] Translation of this tool into a "nano" format might greatly enhance its utility.

Techniques for Relative Protein Quantification at the Discovery Stage of Biomarker Analysis

Biomarker discovery assays are designed to assess differences between the disease and control samples in a quantitative manner.[130-132] In contrast to MRM assays, discovery assays measure relative abundances rather than absolute concentrations. Hence, comparing the levels of the same protein across samples is more reliable than comparing abundances of different proteins in the same sample. Label-free approaches and methods based on the concept of isotopic dilution are commonly used. Label-free methods are attractive because they do not require any specialized sample processing, but they analyze one sample at a time.[133-135] As to the latter approach, a category of isobaric reagents has gained popularity because such reagents offer the opportunity of multiplexing samples for LC MS analysis—thus significantly minimizing analysis time, an important consideration for large proteomics studies. When using isobaric reagents, samples are processed independently up to the stage of labeling proteolytic peptides with stable isotope reagents and then combined for further fractionation and MS.[136,137] An attractive stable isotope–based approach of stable isotope labeling with amino acids in culture (SILAC) was originally introduced for studies on cell cultures where it is possible to incorporate stable isotope–labeled amino acids in vivo as natural precursors of protein synthesis.[138] SILAC allows samples to be combined at much earlier stages of sample preparation, thus minimizing artifacts related to sample processing.[139] Although clinical specimens themselves are not amenable to stable-isotope labeling, generation of labeled reference proteins in cell cultures extended the use of Super-SILAC[140-142] to nonculturable systems.

Specialized Approaches Tailored to Analysis of Protein Glycosylation

Aberrant glycosylation has long been recognized as a hallmark of malignant transformation of mammalian cells,[143,144] and hence changes in protein glycosylation can serve as

sentinels of cancer detection.[145-160] Many of the oldest and most widely used clinical cancer biomarker tests detect glycoproteins, including carcinoembryonic antigen (CEA), used as a marker of colorectal cancer[161,162]; cancer antigen 125 (CA125), frequently used to diagnose ovarian cancer[163,164]; prostate-specific antigen (PSA)[165-167]; and serum CA 19-9, commonly used for the diagnosis of pancreatic cancer.[168,169] However, because of the complexity of carbohydrate structures and related technical challenges, progress in glycoprotein analysis has been slow. Tellingly, the structures of CA125 glycans have not been fully characterized despite more than 30 years of intense investigation.[170] Nevertheless, the past decade saw significant progress in glycomics technologies,[134,167,171-182] including MS methods for direct analysis of glycopeptides[183-186] and development of bioinformatics tools for data analysis.[187-189]

When interrogating the glycoproteome for biomarkers, the proverbial "needle-in-a-haystack" challenge is exacerbated by the great heterogeneity of glycoprotein structures. Different classes of oligosaccharide structures are attached to proteins via Ser/Thr (*O*-linked) and Asn (*N*-linked) residues. Although a consensus sequence for *N*-linked glycosylation is known (NXT/S, where X is any residue except proline), the specific amino acid sequences that make up *O*-glycosylation sites are not well understood. Thus, use of these potential glycosylation sites varies widely for reasons that remain obscure. In addition, there is substantial heterogeneity in the *N*- and *O*-linked glycan motifs across different sites as well as occupancy of individual sites. Analysis of *N*-glycosylation is facilitated by the availability of a specific asparagine deamidase enzyme that releases *N*-linked structures. Peptide *N*-glycosidase F (PNGase F) hydrolyzes the amide bond of the asparagine residue to which the glycan is attached.[190] This process results in a 1-Da increase in the molecular mass of the deglycosylated peptide, thus marking the original *N*-glycosylation site with a mass tag easily identifiable by MS. When performed in the presence of ^{18}O-labeled water,[191] a 3-Da increase in peptide molecular mass provides greater distinction. On the other hand, the development of equivalent enzymes for the release of *O*-linked structures, and identification of their sites of attachment, has yet to be achieved. In this regard, engineering a proteinase that would hydrolyze the GalNAc-Ser/Thr linkage would greatly facilitate studies of *O*-linked structures and the glycoproteins that carry them. Importantly, the HUPO Human Disease Glycomics/Proteome Initiative coordinated a multi-institutional study aimed at evaluating methods for profiling *O*-glycosylation that demonstrated the preeminent performance of MS for *O*-glycopeptide and *O*-glycan analysis.[192] Reliable protocols for characterization of *O*-deglycosylated proteins using chemical[193] or partial enzymatic deglycosylation methods[186] were recently published.

Current approaches to the analysis of glycoproteins as potential biomarkers typically involve one or more enrichment steps, proteolytic digestion, and LC MS analyses. Enrichment of carbohydrate-containing species is performed at either the glycoprotein or glycopeptide level. Although both approaches offer distinct benefits, glycopeptide-based capture provides the advantage of identifying exact protein regions and, often, a specific site of glycosylation. On the other hand, in the workflows based on affinity selection at the glycoprotein level, protein identification is facilitated by the analysis of multiple peptides[194]—although with an increased risk of detecting off-target species, that is, those that either bind to a matrix in a nonspecific manner or interact with glycoprotein targets. The latter phenomenon might prove useful in detecting alterations in protein-protein interactions triggered by cancer-related changes in glycosylation.

Given that affinity selection of glycan-containing molecules is an important component of the glycoproteomics workflow, it is possible to analyze unfractionated samples. However, when analyzing plasma or serum that are overwhelmed by a small number of highly abundant proteins, their immunoaffinity depletion is often done before glycopeptides/protein enrichment, thus enabling deeper coverage of the remaining lower-abundance proteome where biomarkers typically reside. The most commonly used enrichment approaches take advantage of lectins, proteins that specifically bind to various *N*- and *O*-linked carbohydrate motifs.[195,196] In addition, chemical methods that exploit elements of glycan structures have also been introduced.[197]

A variety of protocols have been developed for lectin capture of glycoproteins and glycopeptides, based either on a single lectin[194,198-200] or on a combination of lectins with different selectivities, either in a serial[201] or a mixed-bed multi-lectin affinity capture manner[202-205] and using different analytical formats (e.g., gravity column chromatography, magnetic beads, or high-performance liquid chromatography [HPLC], the last providing the highest degree of reproducibility and speed).[13,14,200,206,207] Elution of captured glycoproteins/glycopeptides is accomplished using either mono- or disaccharides presenting lectin-specific epitopes[200,202,208] or nonspecific disruptors of lectin-glycan binding, such as acidic or high-salt reagents.[194,207,209,210] Additional fractionation of the captured species using various forms of HPLC is often performed before MS analysis. Judicious selection of lectins is vital for the success of a biomarker discovery experiment. An elegant study that explored the performance of lectins of varying specificities highlighted the potential utility of a polylactosamine-directed *Lycopersicon esculentum* lectin (LEL) of narrow specificity in differential analysis of the glycoproteome of breast cancer patients, as compared to control individuals.[194] In a study that focused on *Aleuria aurantia* lectin (AAL) enrichment of highly

fucosylated glycoproteins, approximately 50% of the glycoproteins correlated with a hepatocellular carcinoma diagnosis, with fucosylated hemopexin emerging as a potential biomarker of cancer with a high degree of specificity and selectivity of 92% for both.[199]

As to chemical methods of enrichment, two similar approaches, involving hydrazide[211] or boronic acid chemistry, capitalize on the *cis* diols present in monosaccharide structures. Bound glycoproteins are trypsin-digested in situ, and nonglycosylated/unbound peptides are removed by washing. Then covalently coupled *N*-linked glycopeptides are released by PNGase F treatment and analyzed by LC-MS/MS.[211] In an alternative approach, glycopeptides rather than intact glycoproteins were captured by hydrazide beads,[212] which resulted in a marked increase in the number of recovered glycopeptides. A complementary approach employed boronic acid–functionalized beads to covalently capture glycoproteins, which were then eluted with acid.[213] Building on data derived from these methods, Aebersold's group[214] developed a searchable online catalog for depositing data regarding *N*-linked glycosylation sites and glycopeptide sequences (www.UniPep.org). This repository is an important companion to sequence-based algorithms that predict *N*-linked glycosylation sites and, as such, is a valuable resource for plasma- and serum-based studies.

Studies focused on specific glycan structures rather than on their protein scaffolds are also being aggressively pursued, and a number of glycan-based cancer biomarkers have been proposed.[149,151,158,215-224] Here, glycans are released either by enzymatic (for *N*-linked) or chemical (for *O*-linked) methods, fractionated by chromatography, and analyzed by MS for compositional and structural profiling. The latter approach offers high sensitivity in differentiating between normal and disease-related structures, which are often more elaborate. Recently developed methods use isomer-sensitive stationary phases, such as porous graphitized carbon[220,225] or hydrophilic interaction chromatography,[222,226] for nanoLC separation of complex glycan mixtures. Importantly, high chromatographic resolution and reproducibility of the method allow isomeric structures to be assigned without the need for oligosaccharide derivatization. Recent high-throughput application of this technology to serum samples from ovarian cancer patients (n = 46) and controls (n = 48) revealed approximately 250 *N*-linked glycan features with more than 100 distinct compositions, and provided discrimination between healthy control and cancer samples.[227] Although these results require further validation, they nevertheless speak to the robustness of the platform and its potential utility in a screening format. Ongoing efforts aimed at complete annotation of the "normal" human serum glycome in terms of glycan composition, MS/MS spectra, and LC retention times will greatly facilitate use of platforms and workflows for interrogating the glycoproteome and the glycome spaces, which remain relatively unexplored.[225] Glycan tagging using hydrazide-based chemistry to enhance oligosaccharide ionizability and MS detectability has recently been reviewed.[228] Finally, engineering bioorthogonal monosaccharides bearing biologically inert functional tags that are accepted as natural substrates and incorporated into glycoconjugates is an attractive complementary approach for exploring structures of specific classes of cell surface glycans.[229,230]

Example: Biomarker Development Using a Mouse Model of Human Breast Cancer

A recently published account of credentialing a consolidated set of biomarker candidates in an animal model of breast cancer provides an excellent illustration of the complexity of the process of selecting high-value markers out of the large number of potential contenders.[231] The major focus of this work was verification, which built on the discovery data generated by the authors and other investigators using the results of 13 independent microarray and untargeted shotgun proteomics experiments performed in plasma and tissues. In the course of a multistep verification process, an initial set of 1908 putative biomarkers assembled according to preestablished criteria was reduced to a set of 36 proteins that were verified, using a high-stringency assay, as elevated in the plasma of tumor-bearing mice. From the standpoint of technology, this carefully designed study provides a unique perspective on capabilities of current methods under ideal conditions because the variability related to biological and environmental factors was minimized by using an animal model.

This work used a well-characterized doxycycline-inducible, bitransgenic MMTV-rtTA/TetO-NeuNT (Her2/Neu) mouse model of breast cancer. Healthy transgenic TetO-Neu animals were used as controls. The goal of the first triage step was to identify the candidate biomarkers that were detectable in plasma, as many originated from tissue-based studies. To this end, a targeted MS proteomics method, accurate inclusion mass screening (AIMS),[66] was used to screen pooled plasma from 20 tumor-bearing animals for the presence of peptides representing 1144 candidate proteins. Close to 17,000 proteotypic peptides[232-234]—those that are detectable under the experimental conditions and diagnostic for a given protein—were targeted, resulting in the detection of 43% of the candidate proteins. Not unexpectedly,

the highest confirmation rates were for candidates that originated from plasma analysis. However, 17% of the candidates that were originally discovered only in tissue were identified, including 2% that were derived exclusively from the mRNA analyses. In the second triage step, the authors used a semiquantitative format of SRM to further cull their list of candidate biomarkers. Of 383 candidates that were examined using plasma pools of tumor-bearing and control animals, relative concentrations of 43% and 10% proteins demonstrated differences estimated with high and medium levels of confidence, respectively. The third-stage triage employed quantitative targeted MS assays: SID SRM and SISCAPA. A total of 79 high- and medium-confidence candidates were evaluated in individual animals from two disease cohorts: animals with substantially advanced tumors (cohort 1) or mice in an early phase of the disease process (cohort 2). For cohort 1, 36 proteins (46%) were significantly elevated as compared to disease-free control mice. In contrast, only two proteins circulated at significantly higher levels in the mice from cohort 2 versus controls, which exemplifies the considerable challenge of verifying biomarkers of early-stage tumors. Final verification, which employed wild-type mice with confounding conditions, suggested that most of the identified biomarkers were likely to be specific for breast cancer.

In summary, in addition to its primary value of proposing potential breast cancer biomarkers, the study benchmarks the currently available technologies in a tightly controlled in vivo model. Significantly, of the 369 candidates selected solely on the basis of significant differences in mRNA expression in tissue, only about 2% were confirmed by AIMS, and none were subsequently verified. Likewise, none of the 35 candidates revealed by the label-free proteomics discovery assays in plasma alone were verified. These results clearly demonstrate that biomarker discovery focused solely on plasma is very unlikely to be effective without including concomitant analyses of affected tissues (or proximal fluids) at the protein level.

Conclusions

Currently, many more biomarker candidates have been proposed by MS proteomics discovery studies than have been validated with the rigor required for their use in the clinic. At the same time, only a small portion of the proteome has been interrogated, and hence only a fraction of potential biomarkers have been brought to light. In view of limited resources, this situation poses a dilemma as to what should be prioritized in the near future: verification of the plethora of candidate biomarkers or discovery of new ones. From the perspective of furthering understanding of cancer and helping patients, this is a false choice. Nevertheless, it is bound to affect funding decisions and consequently the directions that the field will take. A clear distinction is required in terms of addressing the needs of the validation versus discovery arms of the biomarker development pipeline. First, a robust strategy for traversing the precarious middle point in candidate biomarker evaluation is required. At the stage of biomarker qualification, the least likely targets need to be weeded out using relatively inexpensive semiquantitative approaches before investing in rigorous and costly assays for the most valuable contenders.[22] Validation requires the analysis of large number of samples from various well-defined cohorts using well-established, well-controlled, uniform, and standardized protocols. From this perspective, large research consortia, with guaranteed access to proper specimens and all the advantages that economy of scale brings in terms of infrastructure, stand the best chance of streamlining the process and producing reliable results in a timely fashion. In addition, major efforts are being made in the biotechnology and biopharmaceutical sectors. Further investments in the field of biomarker discovery should also promote novel protocols and strategies aimed at increasing the breadth and depth of coverage. As history teaches, these types of cutting-edge scientific endeavors tend to be most successful when left to small independent groups of highly creative researchers.

To summarize, we are far from the ability to detect, identify, and quantify every protein in all of its isoforms in biological specimens. When designing experiments, our decisions are based not only on scientific factors, but also on constraints of economy and logistics. Therefore we need to be pragmatic and strive for maximum efficiency while accepting the need for imperfect solutions. This approach goes against the grain of analytical chemistry, creating a healthy tension. In this context, it is more important for biomarker discovery methods to be as comprehensive as possible rather than reproducible, which comes into play at later stages. On the other hand, it is vital for validation methods to be rock solid, artifact free, and reproducible, preferably across analytical platforms and laboratories, to enable generation of the necessarily large volumes of data in a reasonable time frame. In an ideal world, it would be preferable to invest in methods that deliver biomarkers that provide insights into the disease process. However, given the pressing needs of cancer patients and people with other diseases, we are forced to accept less informative solutions that deliver clinically useful biomarkers, in terms of diagnosis and therapeutic responses, regardless of our current understanding of their functional relevance.

Key References

1. Brooks JD. Translational genomics: the challenge of developing cancer biomarkers. *Genome Res*. 2012;22:183-187.
2. Ball P. DNA: Celebrate the unknowns. *Nature*. 2013;496:419-420.
3. Varki A. Omics: Account for the "dark matter" of biology. *Nature*. 2013;497:565.
4. Mischak H, Ioannidis JP, Argiles A, et al. Implementation of proteomic biomarkers: making it work. *Eur J Clin Invest*. 2012;42:1027-1036.
5. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422:198-207.
6. Check E. Proteomics and cancer: running before we can walk? *Nature*. 2004; 429:496-497.
7. Master SR. Diagnostic proteomics: back to basics? *Clin Chem*. 2005; 51:1333-1334.
8. Ransohoff DF. Lessons from controversy: ovarian cancer screening and serum proteomics. *J Natl Cancer Inst*. 2005;97:315-319.
9. Addona TA, Abbatiello SE, Schilling B, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol*. 2009;27:633-641.
10. Gloriam DE, Orchard S, Bertinetti D, et al. A community standard format for the representation of protein affinity reagents. *Mol Cell Proteomics*. 2010;9:1-10.
11. Paulovich AG, Billheimer D, Ham AJ, et al. Interlaboratory study characterizing a yeast performance standard for benchmarking LC-MS platform performance. *Mol Cell Proteomics*. 2010;9:242-254.
12. Rudnick PA, Clauser KR, Kilpatrick LE, et al. Performance metrics for liquid chromatography-tandem mass spectrometry systems in proteomics analyses. *Mol Cell Proteomics*. 2010;9:225-241.
13. Drake PM, Schilling B, Niles RK, et al. A lectin affinity workflow targeting glycosite-specific, cancer-related carbohydrate structures in trypsin-digested human plasma. *Anal Biochem*. 2011;408:71-85.
14. Drake PM, Schilling B, Niles RK, et al. Lectin chromatography/mass spectrometry discovery workflow identifies putative biomarkers of aggressive breast cancers. *J Proteome Res*. 2012;11:2508-2520.
15. Tabb DL, Vega-Montoto L, Rudnick PA, et al. Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. *J Proteome Res*. 2010;9:761-776.
16. Gaudet P, Argoud-Puy G, Cusin I, et al. neXtProt: organizing protein knowledge in the context of human proteome projects. *J Proteome Res*. 2013;12:293-298.
17. Hamacher M, Grottrup B, Eisenacher M, et al. Inter-lab proteomics: data mining in collaborative projects on the basis of the HUPO brain proteome project's pilot studies. *Methods Mol Biol*. 2011;696:235-246.
18. Legrain P, Aebersold R, Archakov A, et al. The human proteome project: current state and future direction. *Mol Cell Proteomics*. 2011.
19. Orchard S. Molecular interaction databases. *Proteomics*. 2012;12:1656-1662.
20. Stoevesandt O, Taussig MJ. European and international collaboration in affinity proteomics. *Nat Biotechnol*. 2012;29:511-514.
21. Lamond AI, Uhlen M, Horning S, et al. Advancing cell biology through proteomics in space and time (PROSPECTS). *Mol Cell Proteomics*. 2012;11: O112.017731.
22. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol*. 2006;24:971-983.
23. Cox J, Mann M. Is proteomics the new genomics? *Cell*. 2007;130:395-398.
24. Surinova S, Schiess R, Huttenhain R, Cerciello F, Wollscheid B, Aebersold R. On the development of plasma protein biomarkers. *J Proteome Res*. 2011;10:5-16.
25. Taylor CF, Hermjakob H, Julian Jr RK, Garavelli JS, Aebersold R, Apweiler R. The work of the Human Proteome Organisation's Proteomics Standards Initiative (HUPO PSI). *OMICS*. 2006;10:145-151.
26. Taylor CF, Paton NW, Lilley KS, et al. The minimum information about a proteomics experiment (MIAPE). *Nat Biotechnol*. 2007;25:887-893.
28. Kinsinger CR, Apffel J, Baker M, et al. Recommendations for mass spectrometry data quality metrics for open access data (corollary to the Amsterdam principles). *Proteomics Clin App*. 2011;5:580-589.
29. Tan HT, Lee YH, Chung MC. Cancer proteomics. *Mass Spectrom Rev*. 2012;31:583-605.
30. Rodriguez H, Snyder M, Uhlen M, et al. Recommendations from the 2008 International Summit on Proteomics Data Release and Sharing Policy: the Amsterdam principles. *J Proteome Res*. 2009;8:3689-3692.
31. Anderson JF, Siller E, Barral JM. Disorders of protein biogenesis and stability. *Protein Pept Lett*. 2011;18:110-121.
32. Copley SD. Moonlighting is mainstream: paradigm adjustment required. *Bioessays*. 2012;34:578-588.
33. Gidalevitz T, Prahlad V, Morimoto RI. The stress of protein misfolding: from single cells to multicellular organisms. *Cold Spring Harb Perspect Biol*. 2011;3: pii:a009704.
34. Nibbe RK, Chowdhury SA, Koyuturk M, Ewing R, Chance MR. Protein-protein interaction networks and subnetworks in the biology of disease. *Wiley Interdiscip Rev Syst Biol Med*. 2011;3:357-367.
35. Doucet A, Butler GS, Rodriguez D, Prudova A, Overall CM. Metadegradomics: toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome. *Mol Cell Proteomics*. 2008;7:1925-1951.
36. Meshorer E, Misteli T. Splicing misplaced. *Cell*. 2005;122:317-318.
37. Pakharukova NA, Pastushkova L, Moshkovskii SA, Larina IM. [Variability of healthy human proteome]. *Biomed Khim*. 2012;58:514-529.
38. Altshuler DM, Gibbs RA, Peltonen L, et al. Integrating common and rare genetic variation in diverse human populations. *Nature*. 2010;467:52-58.
39. Imanishi T, Itoh T, Suzuki Y, et al. Integrative annotation of 21,037 human genes validated by full-length cDNA clones. *PLoS Biol*. 2004;2:e162.
40. Kersey P, Bower L, Morris L, et al. Integr8 and Genome Reviews: integrated views of complete genomes and proteomes. *Nucleic Acids Res*. 2005;33:D297-D302.
41. Pruess M, Kersey P, Kulikova T, Apweiler R. Databases and resources for in silico proteome analysis. *Methods Biochem Anal*. 2006;49:397-414.
42. Omenn GS, States DJ, Adamski M, et al. Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics*. 2005;5:3226-3245.
43. Zhang Q, Menon R, Deutsch EW, et al. A mouse plasma peptide atlas as a resource for disease proteomics. *Genome Biol*. 2008;9:R93.
44. Kamath KS, Vasavada MS, Srivastava S. Proteomic databases and tools to decipher post-translational modifications. *J Proteomics*. 2011;75:127-144.
45. Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW. Population proteomics: the concept, attributes, and potential for cancer biomarker research. *Mol Cell Proteomics*. 2006;5:1811-1818.
46. Asplund A, Edqvist PH, Schwenk JM, Ponten F. Antibodies for profiling the human proteome—The Human Protein Atlas as a resource for cancer research. *Proteomics*. 2012;12:2067-2077.
47. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248-1250.
48. Fry S, Afrough B, Leathem A, Dwek M. Lectin array-based strategies for identifying metastasis-associated changes in glycosylation. *Methods Mol Biol*. 2012;878:267-272.
49. Oliveira C, Teixeira JA, Domingues L. Recombinant lectins: an array of tailor-made glycan-interaction biosynthetic tools. *Crit Rev Biotechnol*. 2013;33:66-80.
50. Nagaraj N, Mann M. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. *J Proteome Res*. 2011;10:637-645.
51. Moreira JM, Cabezon T, Gromova I, et al. Tissue proteomics of the human mammary gland: towards an abridged definition of the molecular phenotypes underlying epithelial normalcy. *Mol Oncol*. 2010;4:539-561.
52. Ghosh SK, Yohannes E, Bebek G, et al. Proteomic and bioinformatic profile of primary human oral epithelial cells. *J Proteome Res*. 2012;11:5492-5502.
53. Zhou HJ, Liu YK, Chui JF, et al. A glycoproteome database of normal human liver tissue. *J Cancer Res Clin Oncol*. 2007;133:379-387.
54. Elortza F, Beretta L, Zhang X, He F, Mato JM. The 2011 Human Liver Proteome Project (HLPP) Workshop September 5, 2011, Geneva, Switzerland. *Proteomics*. 2012;12:5-8.

55. Grottrup B, Marcus K, Grinberg LT, Lee SK, Meyer HE, Park YM. Creating a human brain proteome atlas—14th HUPO BPP workshop September 20-21, 2010, Sydney, Australia. *Proteomics*. 2011;11:3269-3272.
56. Veenstra TD. Proteome analysis of posttranslational modifications. *Adv Protein Chem*. 2003;65:161-194.
60. Schmidt A, Claassen M, Aebersold R. Directed mass spectrometry: towards hypothesis-driven proteomics. *Curr Opin Chem Biol*. 2009;13:510-517.
61. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877:1229-1239.
62. Shi T, Su D, Liu T, et al. Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics*. 2012;12:1074-1092.
63. Boja ES, Rodriguez H. Mass spectrometry-based targeted quantitative proteomics: achieving sensitive and reproducible detection of proteins. *Proteomics*. 2012;12:1093-1110.
64. Meng Z, Veenstra TD. Targeted mass spectrometry approaches for protein biomarker verification. *J Proteomics*. 2011;74:2650-2659.
65. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*. 2004;3:235-244.
66. Jaffe JD, Keshishian H, Chang B, Addona TA, Gillette MA, Carr SA. Accurate inclusion mass screening: a bridge from unbiased discovery to targeted assay development for biomarker verification. *Mol Cell Proteomics*. 2008;7:1952-1962.
67. Smith RD, Anderson GA, Lipton MS, et al. An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics*. 2002;2:513-523.
70. Catherman AD, Li M, Tran JC, et al. Top down proteomics of human membrane proteins from enriched mitochondrial fractions. *Anal Chem*. 2013;85:1880-1888.
71. Michalski A, Damoc E, Lange O, et al. Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Mol Cell Proteomics*. 2012;11: O111.013698.
72. Young NL, Plazas-Mayorca MD, DiMaggio PA, et al. Collective mass spectrometry approaches reveal broad and combinatorial modification of high mobility group protein A1a. *J Am Soc Mass Spectrom*. 2010;21:960-970.
73. Frahm JL, Howard BE, Heber S, Muddiman DC. Accessible proteomics space and its implications for peak capacity for zero-, one- and two-dimensional separations coupled with FT-ICR and TOF mass spectrometry. *J Mass Spectrom*. 2006;41:281-288.
74. Sandhu C, Hewel JA, Badis G, et al. Evaluation of data-dependent versus targeted shotgun proteomic approaches for monitoring transcription factor expression in breast cancer. *J Proteome Res*. 2008;7:1529-1541.
75. Juhasz P, Lynch M, Sethuraman M, et al. Semi-targeted plasma proteomics discovery workflow utilizing two-stage protein depletion and off-line LC-MALDI MS/MS. *J Proteome Res*. 2011;10:34-45.
76. Savitski MM, Fischer F, Mathieson T, Sweetman G, Lang M, Bantscheff M. Targeted data acquisition for improved reproducibility and robustness of proteomic mass spectrometry assays. *J Am Soc Mass Spectrom*. 2010;21:1668-1679.
77. Wang N, Li L. Exploring the precursor ion exclusion feature of liquid chromatography–electrospray ionization quadrupole time-of-flight mass spectrometry for improving protein identification in shotgun proteome analysis. *Anal Chem*. 2008;80:4696-4710.
78. Yokosuka T, Yoshinari K, Kobayashi K, et al. "Information-Based-Acquisition" (IBA) technique with an ion-trap/time-of-flight mass spectrometer for high-throughput and reliable protein profiling. *Rapid Commun Mass Spectrom*. 2006;20:2589-2595.
79. Zerck A, Nordhoff E, Resemann A, et al. An iterative strategy for precursor ion selection for LC-MS/MS based shotgun proteomics. *J Proteome Res*. 2009;8:3239-3251.
80. Graumann J, Scheltema RA, Zhang Y, Cox J, Mann M. A framework for intelligent data acquisition and real-time database searching for shotgun proteomics. *Mol Cell Proteomics*. 2012;11: M111.013185.
81. Liu H, Yang L, Khainovski N, et al. Automated iterative MS/MS acquisition: a tool for improving efficiency of protein identification using a LC-MALDI MS workflow. *Anal Chem*. 2011;83:6286-6293.
82. Nesvizhskii AI. Protein identification by tandem mass spectrometry and sequence database searching. *Methods Mol Biol*. 2007;367:87-119.
83. Bromenshenk JJ, Henderson CB, Wick CH, et al. Iridovirus and microsporidian linked to honey bee colony decline. *PLoS One*. 2010;5:e13181.
84. Knudsen GM, Chalkley RJ. The effect of using an inappropriate protein database for proteomic data analysis. *PLoS One*. 2011;6:e20873.
85. Albrethsen J. The first decade of MALDI protein profiling: a lesson in translational biomarker research. *J Proteomics*. 2011;74:765-773.
86. Kuwata H, Yip TT, Yip CL, Tomita M, Hutchens TW. Bactericidal domain of lactoferrin: detection, quantitation, and characterization of lactoferricin in serum by SELDI affinity mass spectrometry. *Biochem Biophys Res Commun*. 1998;245:764-773.
87. Merchant M, Weinberger SR. Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis*. 2000;21:1164-1177.
88. Szajli E, Feher T, Medzihradszky KF. Investigating the quantitative nature of MALDI-TOF MS. *Mol Cell Proteomics*. 2008;7:2410-2418.
89. Fung ET. A recipe for proteomics diagnostic test development: the OVA1 test, from biomarker discovery to FDA clearance. *Clin Chem*. 2010;56:327-329.
90. Exploring FDA-approved IVDMIAs; 2011. Retrieved from http://www.ivdtechnology.com/article/exploring-fda-approved-ivdmias.
91. Moore LE, Pfeiffer RM, Zhang Z, Lu KH, Fung ET, Bast Jr RC. Proteomic biomarkers in combination with CA 125 for detection of epithelial ovarian cancer using prediagnostic serum samples from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. *Cancer*. 2012;118:91-100.
92. Callesen AK, Mogensen O, Jensen AK, et al. Reproducibility of mass spectrometry based protein profiles for diagnosis of ovarian cancer across clinical studies: a systematic review. *J Proteomics*. 2012;75:2758-2772.
93. Purvine S, Eppel JT, Yi EC, Goodlett DR. Shotgun collision-induced dissociation of peptides using a time of flight mass analyzer. *Proteomics*. 2003;3:847-850.
94. Geromanos SJ, Vissers JP, Silva JC, et al. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependent LC-MS/MS. *Proteomics*. 2009;9:1683-1695.
95. Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics*. 2006;5:144-156.
96. Gillet LC, Navarro P, Tate S, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics*. 2012;11: O111.016717.
97. Liu Y, Huttenhain R, Surinova S, et al. Quantitative measurements of N-linked glycoproteins in human plasma by SWATH-MS. *Proteomics*. 2013;13:1247-1256.
98. Horvatovich P, Hoekman B, Govorukhina N, Bischoff R. Multidimensional chromatography coupled to mass spectrometry in analysing complex proteomics samples. *J Sep Sci*. 2010;33:1421-1437.
99. Angel TE, Aryal UK, Hengel SM, et al. Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem Soc Rev*. 2012;41:3912-3928.
100. Harvey SR, Macphee CE, Barran PE. Ion mobility mass spectrometry for peptide analysis. *Methods*. 2011;54:454-461.
101. Valentine SJ, Liu X, Plasencia MD, et al. Developing liquid chromatography ion mobility mass spectrometry techniques. *Expert Rev Proteomics*. 2005;2:553-565.
102. Kalnina Z, Zayakin P, Silina K, Line A. Alterations of pre-mRNA splicing in cancer. *Genes Chromosomes Cancer*. 2005;42:342-357.
103. Pajares MJ, Ezponda T, Catena R, Calvo A, Pio R, Montuenga LM. Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol*. 2007;8:349-357.
104. Omenn GS, Yocum AK, Menon R. Alternative splice variants, a new class of protein cancer biomarker candidates: findings in pancreatic cancer and breast cancer with systems biology implications. *Dis Markers*. 2010;28:241-251.
105. Kaida D, Schneider-Poetsch T, Yoshida M. Splicing in oncogenesis and tumor suppression. *Cancer Sci*. 2012;103:1611-1616.

106. Ferreira EN, Galante PA, Carraro DM, de Souza SJ. Alternative splicing: a bioinformatics perspective. *Mol Biosyst*. 2007;3:473-477.
107. Power KA, McRedmond JP, de Stefani A, Gallagher WM, Gaora PO. High-throughput proteomics detection of novel splice isoforms in human platelets. *PLoS One*. 2009;4:e5001.
108. Menon R, Omenn GS. Proteomic characterization of novel alternative splice variant proteins in human epidermal growth factor receptor 2/neu-induced breast cancers. *Cancer Res*. 2010;70:3440-3449.
109. Brosch M, Saunders GI, Frankish A, et al. Shotgun proteomics aids discovery of novel protein-coding genes, alternative splicing, and "resurrected" pseudogenes in the mouse genome. *Genome Res*. 2011;21:756-767.
110. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics*. 2006;5:573-588.
111. Qian WJ, Kaleta DT, Petritis BO, et al. Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol Cell Proteomics*. 2008;7:1963-1973.
112. Freeman WM, Lull ME, Guilford MT, Vrana KE. Depletion of abundant proteins from non-human primate serum for biomarker studies. *Proteomics*. 2006;6:3109-3113.
113. Gong Y, Li X, Yang B, et al. Different immunoaffinity fractionation strategies to characterize the human plasma proteome. *J Proteome Res*. 2006;5:1379-1387.
114. Polaskova V, Kapur A, Khan A, Molloy MP, Baker MS. High-abundance protein depletion: comparison of methods for human plasma biomarker discovery. *Electrophoresis*. 2010;31:471-482.
115. Yadav AK, Bhardwaj G, Basak T, et al. A systematic analysis of eluted fraction of plasma post immunoaffinity depletion: implications in biomarker discovery. *PLoS One*. 2011;6:e24442.
116. Whiteaker JR, Zhang H, Eng JK, et al. Head-to-head comparison of serum fractionation techniques. *J Proteome Res*. 2007;6:828-836.
117. Bellei E, Bergamini S, Monari E, et al. High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins. *Amino Acids*. 2011;40:145-156.
118. Camaggi CM, Zavatto E, Gramantieri L, et al. Serum albumin-bound proteomic signature for early detection and staging of hepatocarcinoma: sample variability and data classification. *Clin Chem Lab Med*. 2010;48:1319-1326.
119. Gundry RL, Fu Q, Jelinek CA, Van Eyk JE, Cotter RJ. Investigation of an albumin-enriched fraction of human serum and its albuminome. *Proteomics Clin App*. 2007;1:73-88.
120. Scumaci D, Gaspari M, Saccomanno M, et al. Assessment of an ad hoc procedure for isolation and characterization of human albuminome. *Anal Biochem*. 2011;418:161-163.
121. Koutroukides TA, Guest PC, Leweke FM, et al. Characterization of the human serum depletome by label-free shotgun proteomics. *J Sep Sci*. 2011;34:1621-1626.
122. Sennels L, Salek M, Lomas L, Boschetti E, Righetti PG, Rappsilber J. Proteomic analysis of human blood serum using peptide library beads. *J Proteome Res*. 2007;6:4055-4062.
123. Thulasiraman V, Lin S, Gheorghiu L, et al. Reduction of the concentration difference of proteins in biological liquids using a library of combinatorial ligands. *Electrophoresis*. 2005;26:3561-3571.
124. Roux-Dalvai F, Gonzalez de Peredo A, Simo C, et al. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol Cell Proteomics*. 2008;7:2254-2269.
125. Ernoult E, Bourreau A, Gamelin E, Guette C. A proteomic approach for plasma biomarker discovery with iTRAQ labelling and OFFGEL fractionation. *J Biomed Biotechnol*. 2010;2010:927917.
126. Colzani M, Waridel P, Laurent J, Faes E, Ruegg C, Quadroni M. Metabolic labeling and protein linearization technology allow the study of proteins secreted by cultured cells in serum-containing media. *J Proteome Res*. 2009;8:4779-4788.
127. Bandhakavi S, Van Riper SK, Tawfik PN, et al. Hexapeptide libraries for enhanced protein PTM identification and relative abundance profiling in whole human saliva. *J Proteome Res*. 2012;10:1052-1061.
130. Elliott MH, Smith DS, Parker CE, Borchers C. Current trends in quantitative proteomics. *J Mass Spectrom*. 2009;44:1637-1660.
131. Liang S, Xu Z, Xu X, Zhao X, Huang C, Wei Y. Quantitative proteomics for cancer biomarker discovery. *Comb Chem High Throughput Screen*. 2012;15:221-231.
132. Rajcevic U, Niclou SP, Jimenez CR. Proteomics strategies for target identification and biomarker discovery in cancer. *Front Biosci*. 2009;14:3292-3303.
133. Neilson KA, Ali NA, Muralidharan S, et al. Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics*. 2011;11:535-553.
134. Rebecchi KR, Wenke JL, Go EP, Desaire H. Label-free quantitation: a new glycoproteomics approach. *J Am Soc Mass Spectrom*. 2009;20:1048-1059.
135. Zhou W, Liotta LA, Petricoin EF. The spectra count label-free quantitation in cancer proteomics. *Cancer Genomics Proteomics*. 2012;9:135-142.
136. Ross PL, Huang YN, Marchese JN, et al. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics*. 2004;3:1154-1169.
137. Thompson A, Schafer J, Kuhn K, et al. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem*. 2003;75:1895-1904.
139. Kashyap MK, Harsha HC, Renuse S, et al. SILAC-based quantitative proteomic approach to identify potential biomarkers from the esophageal squamous cell carcinoma secretome. *Cancer Biol Ther*. 2010;10:796-810.
140. Boersema PJ, Geiger T, Wisniewski JR, Mann M. Quantification of the N-glycosylated secretome by super-SILAC during breast cancer progression and in human blood samples. *Mol Cell Proteomics*. 2013;12:158-171.
141. Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M. Super-SILAC mix for quantitative proteomics of human tumor tissue. *Nat Methods*. 2010;7:383-385.
142. Lund RR, Terp MG, Laenkholm AV, Jensen ON, Leth-Larsen R, Ditzel HJ. Quantitative proteomics of primary tumors with varying metastatic capabilities using stable isotope-labeled proteins of multiple histogenic origins. *Proteomics*. 2012;12:2139-2148.
145. de Leoz ML, Young LJ, An HJ, et al. High-mannose glycans are elevated during breast cancer progression. *Mol Cell Proteomics*. 2011;10: M110.002717.
146. Comunale MA, Wang M, Rodemich-Betesh L, et al. Novel changes in glycosylation of serum Apo-J in patients with hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1222-1229.
147. Ahn YH, Lee JY, Kim YS, Ko JH, Yoo JS. Quantitative analysis of an aberrant glycoform of TIMP1 from colon cancer serum by L-PHA-enrichment and SISCAPA with MRM mass spectrometry. *J Proteome Res*. 2009;8:4216-4224.
148. Li B, An HJ, Kirmiz C, Lebrilla CB, Lam KS, Miyamoto S. Glycoproteomic analyses of ovarian cancer cell lines and sera from ovarian cancer patients show distinct glycosylation changes in individual proteins. *J Proteome Res*. 2008;7:3776-3788.
149. Leiserowitz GS, Lebrilla C, Miyamoto S, et al. Glycomics analysis of serum: a potential new biomarker for ovarian cancer? *Int J Gynecol Cancer*. 2008;18:470-475.
150. Kyselova Z, Mechref Y, Kang P, et al. Breast cancer diagnosis and prognosis through quantitative measurements of serum glycan profiles. *Clin Chem*. 2008;54:1166-1175.
151. Kirmiz C, Li B, An HJ, et al. A serum glycomics approach to breast cancer biomarkers. *Mol Cell Proteomics*. 2007;6:43-55.
152. Ahn YH, Kim KH, Shin PM, Ji ES, Kim H, Yoo JS. Identification of low-abundance cancer biomarker candidate TIMP1 from serum with lectin fractionation and peptide affinity enrichment by ultrahigh-resolution mass spectrometry. *Anal Chem*. 2012;84:1425-1431.
153. Drake PM, Cho W, Li B, et al. Sweetening the pot: adding glycosylation to the biomarker discovery equation. *Clin Chem*. 2010;56:223-236.
154. Dube DH, Bertozzi CR. Glycans in cancer and inflammation–potential for therapeutics and diagnostics. *Nat Rev Drug Discov*. 2005;4:477-488.
155. Kim EH, Misek DE. Glycoproteomics-based identification of cancer biomarkers. *Int J Proteomics*. 2011;2011:601937. doi: 10.1155/2011/601937. Epub 2011 Sep 28.
156. Ruhaak LR, Miyamoto S, Lebrilla CB. Developments in the identification of glycan biomarkers for the detection of cancer. *Mol Cell Proteomics*. 2013;12:146-155.
157. Angata T, Fujinawa R, Kurimoto A, et al. Integrated approach toward the discovery of glyco-biomarkers of inflammation-related diseases. *Ann N Y Acad Sci*. 2012;1253:159-169.

158. Adamczyk B, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochim Biophys Acta*. 2012;1820:1347-1353.
159. Lee A, Chick JM, Kolarich D, et al. Liver membrane proteome glycosylation changes in mice bearing an extra-hepatic tumor. *Mol Cell Proteomics*. 2011;10: M900538MCP200. doi: 10.1074/mcp.M900538-MCP200. Epub 2010 Feb 18.
160. Balog CI, Stavenhagen K, Fung WL, et al. N-glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. *Mol Cell Proteomics*. 2012;11:571-585.
162. van Gisbergen KP, Aarnoudse CA, Meijer GA, Geijtenbeek TB, van Kooyk Y. Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin. *Cancer Res*. 2005;65:5935-5944.
163. Jankovic MM, Milutinovic BS. Glycoforms of CA125 antigen as a possible cancer marker. *Cancer Biomark*. 2008;4:35-42.
164. Moss EL, Hollingworth J, Reynolds TM. The role of CA125 in clinical practice. *J Clin Pathol*. 2005;58:308-312.
165. Meany DL, Zhang Z, Sokoll LJ, Zhang H, Chan DW. Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. *J Proteome Res*. 2009;8:613-619.
166. Ohyama C, Hosono M, Nitta K, et al. Carbohydrate structure and differential binding of prostate specific antigen to *Maackia amurensis* lectin between prostate cancer and benign prostate hypertrophy. *Glycobiology*. 2004;14:671-679.
167. Vermassen T, Speeckaert MM, Lumen N, Rottey S, Delanghe JR. Glycosylation of prostate specific antigen and its potential diagnostic applications. *Clin Chim Acta*. 2012;413:1500-1505.
170. Weiland F, Martin K, Oehler MK, Hoffmann P. Deciphering the molecular nature of ovarian cancer biomarker CA125. *Int J Mol Sci*. 2012;13:10568-10582.
171. Vanderschaeghe D, Festjens N, Delanghe J, Callewaert N. Glycome profiling using modern glycomics technology: technical aspects and applications. *Biol Chem*. 2010;391:149-161.
172. Mahal LK. Glycomics: towards bioinformatic approaches to understanding glycosylation. *Anticancer Agents Med Chem*. 2008;8:37-51.
173. Desaire H. Glycopeptide analysis: recent developments and applications. *Mol Cell Proteomics*. 2013;12:893-901.
174. Mechref Y, Hu Y, Garcia A, Hussein A. Identifying cancer biomarkers by mass spectrometry-based glycomics. *Electrophoresis*. 2012;33:1755-1767.
175. Pan S, Chen R, Aebersold R, Brentnall TA. Mass spectrometry based glycoproteomics—from a proteomics perspective. *Mol Cell Proteomics*. 2011;10: R110.003251.
176. Rakus JF, Mahal LK. New technologies for glycomic analysis: toward a systematic understanding of the glycome. *Annu Rev Anal Chem (Palo Alto Calif)*. 2011;4:367-392.
177. Kuzmanov U, Kosanam H, Diamandis EP. The sweet and sour of serological glycoprotein tumor biomarker quantification. *BMC Med*. 2013;11:31.
178. Wuhrer M. Glycomics using mass spectrometry. *Glycoconj J*. 2013;30:11-22.
179. Narimatsu H, Sawaki H, Kuno A, Kaji H, Ito H, Ikehara Y. A strategy for discovery of cancer glyco-biomarkers in serum using newly developed technologies for glycoproteomics. *FEBS J*. 2010;277:95-105.
180. Patwa T, Li C, Simeone DM, Lubman DM. Glycoprotein analysis using protein microarrays and mass spectrometry. *Mass Spectrom Rev*. 2010;29: 830-844.
181. Zhang Y, Yin H, Lu H. Recent progress in quantitative glycoproteomics. *Glycoconj J*. 2012;29:249-258.
182. Pasing Y, Sickmann A, Lewandrowski U. N-glycoproteomics: mass spectrometry-based glycosylation site annotation. *Biol Chem*. 2012;393:249-258.
183. Saba J, Dutta S, Hemenway E, Viner R. Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int J Proteomics*. 2012;2012:560391.
184. Hanisch FG. O-glycoproteomics: site-specific O-glycoprotein analysis by CID/ETD electrospray ionization tandem mass spectrometry and top-down glycoprotein sequencing by in-source decay MALDI mass spectrometry. *Methods Mol Biol*. 2012;842:179-189.
185. Wang H, Wong CH, Chin A, et al. Integrated mass spectrometry-based analysis of plasma glycoproteins and their glycan modifications. *Nat Protoc*. 2011;6:253-269.
186. Darula Z, Sherman J, Medzihradszky KF. How to dig deeper? Improved enrichment methods for mucin core-1 type glycopeptides. *Mol Cell Proteomics*. 2012;11: O111.016774.
187. von der Lieth CW, Bohne-Lang A, Lohmann KK, Frank M. Bioinformatics for glycomics: status, methods, requirements and perspectives. *Brief Bioinform*. 2004;5:164-178.
188. von der Lieth CW, Lutteke T, Frank M. The role of informatics in glycobiology research with special emphasis on automatic interpretation of MS spectra. *Biochim Biophys Acta*. 2006;1760:568-577.
189. Apte A, Meitei NS. Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan. *Methods Mol Biol*. 2010;600:269-281.
191. Kaji H, Isobe T. Stable isotope labeling of N-glycosylated peptides by enzymatic deglycosylation for mass spectrometry-based glycoproteomics. *Methods Mol Biol*. 2013;951:217-227.
192. Wada Y, Dell A, Haslam SM, et al. Comparison of methods for profiling O-glycosylation: Human Proteome Organisation Human Disease Glycomics/Proteome Initiative multi-institutional study of IgA1. *Mol Cell Proteomics*. 2010;9:719-727.
193. Hanisch FG. Chemical de-O-glycosylation of glycoproteins for applications in LC-based proteomics. *Methods Mol Biol*. 2011;753:323-333.
194. Jung K, Cho W, Regnier FE. Glycoproteomics of plasma based on narrow selectivity lectin affinity chromatography. *J Proteome Res*. 2009;8:643-650.
195. Abbott KL, Pierce JM. Lectin-based glycoproteomic techniques for the enrichment and identification of potential biomarkers. *Methods Enzymol*. 2010;480:461-476.
196. Fanayan S, Hincapie M, Hancock WS. Using lectins to harvest the plasma/serum glycoproteome. *Electrophoresis*. 2012;33:1746-1754.
197. Bond MR, Kohler JJ. Chemical methods for glycoprotein discovery. *Curr Opin Chem Biol*. 2007;11:52-58.
199. Comunale MA, Wang M, Hafner J, et al. Identification and development of fucosylated glycoproteins as biomarkers of primary hepatocellular carcinoma. *J Proteome Res*. 2009;8:595-602.
200. Xiong L, Andrews D, Regnier F. Comparative proteomics of glycoproteins based on lectin selection and isotope coding. *J Proteome Res*. 2003;2:618-625.
201. Durham M, Regnier FE. Targeted glycoproteomics: serial lectin affinity chromatography in the selection of O-glycosylation sites on proteins from the human blood proteome. *J Chromatogr A*. 2006;1132:165-173.
202. Calvano CD, Zambonin CG, Jensen ON. Assessment of lectin and HILIC based enrichment protocols for characterization of serum glycoproteins by mass spectrometry. *J Proteomics*. 2008;71:304-317.
203. Heo SH, Lee SJ, Ryoo HM, Park JY, Cho JY. Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. *Proteomics*. 2007;7:4292-4302.
204. Yang Z, Harris LE, Palmer-Toy DE, Hancock WS. Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. *Clin Chem*. 2006;52:1897-1905.
205. Plavina T, Wakshull E, Hancock WS, Hincapie M. Combination of abundant protein depletion and multi-lectin affinity chromatography (M-LAC) for plasma protein biomarker discovery. *J Proteome Res*. 2007;6:662-671.
206. Kullolli M, Hancock WS, Hincapie M. Preparation of a high-performance multi-lectin affinity chromatography (HP-M-LAC) adsorbent for the analysis of human plasma glycoproteins. *J Sep Sci*. 2008;31:2733-2739.
207. Drake P, Schilling B, Gibson B, Fisher S. Elucidation of N-glycosites within human plasma glycoproteins for cancer biomarker discovery. *Methods Mol Biol*. 2013;951:307-322.
208. Yang Z, Hancock WS. Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column. *J Chromatogr A*. 2004;1053:79-88.
209. Abbott KL, Aoki K, Lim JM, et al. Targeted glycoproteomic identification of biomarkers for human breast carcinoma. *J Proteome Res*. 2008;7:1470-1480.
210. Cho W, Jung K, Regnier FE. Use of glycan targeting antibodies to identify cancer-associated glycoproteins in plasma of breast cancer patients. *Anal Chem*. 2008;80:5286-5292.
211. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat Biotechnol*. 2003;21:660-666.

212. Tian Y, Zhou Y, Elliott S, Aebersold R, Zhang H. Solid-phase extraction of N-linked glycopeptides. *Nat Protoc*. 2007;2:334-339.
213. Sparbier K, Asperger A, Resemann A, et al. Analysis of glycoproteins in human serum by means of glycospecific magnetic bead separation and LC-MALDI-TOF/TOF analysis with automated glycopeptide detection. *J Biomol Tech*. 2007;18:252-258.
214. Zhang H, Loriaux P, Eng J, et al. UniPep—a database for human N-linked glycosites: a resource for biomarker discovery. *Genome Biol*. 2006;7:R73.
215. An HJ, Miyamoto S, Lancaster KS, et al. Profiling of glycans in serum for the discovery of potential biomarkers for ovarian cancer. *J Proteome Res*. 2006;5:1626-1635.
216. de Leoz ML, An HJ, Kronewitter S, et al. Glycomic approach for potential biomarkers on prostate cancer: profiling of N-linked glycans in human sera and pRNS cell lines. *Dis Markers*. 2008;25:243-258.
217. Bereman MS, Williams TI, Muddiman DC. Development of a nanoLC LTQ orbitrap mass spectrometric method for profiling glycans derived from plasma from healthy, benign tumor control, and epithelial ovarian cancer patients. *Anal Chem*. 2009;81:1130-1136.
218. Bereman MS, Young DD, Deiters A, Muddiman DC. Development of a robust and high throughput method for profiling N-linked glycans derived from plasma glycoproteins by NanoLC-FTICR mass spectrometry. *J Proteome Res*. 2009;8:3764-3770.
219. Tang Z, Varghese RS, Bekesova S, et al. Identification of N-glycan serum markers associated with hepatocellular carcinoma from mass spectrometry data. *J Proteome Res*. 2010;9:104-112.
220. Hua S, An HJ, Ozcan S, et al. Comprehensive native glycan profiling with isomer separation and quantitation for the discovery of cancer biomarkers. *Analyst*. 2011;136:3663-3671.
221. Alley Jr WR, Vasseur JA, Goetz JA, et al. N-linked glycan structures and their expressions change in the blood sera of ovarian cancer patients. *J Proteome Res*. 2012;11:2282-2300.
222. Bones J, Mittermayr S, O'Donoghue N, Guttman A, Rudd PM. Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. *Anal Chem*. 2010;82:10208-10215.
223. Hua S, Lebrilla C, An HJ. Application of nano-LC-based glycomics towards biomarker discovery. *Bioanalysis*. 2011;3:2573-2585.
224. Isailovic D, Plasencia MD, Gaye MM, et al. Delineating diseases by IMS-MS profiling of serum N-linked glycans. *J Proteome Res*. 2012;11:576-585.
225. Aldredge D, An HJ, Tang N, Waddell K, Lebrilla CB. Annotation of a serum N-glycan library for rapid identification of structures. *J Proteome Res*. 2012;11:1958-1968.
226. Wuhrer M, de Boer AR, Deelder AM. Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry. *Mass Spectrom Rev*. 2009;28:192-206.
227. Hua S, Williams CC, Dimapasoc LM, et al. Isomer-specific chromatographic profiling yields highly sensitive and specific potential N-glycan biomarkers for epithelial ovarian cancer. *J Chromatogr A*. 2013;1279:58-67.
228. Lattová E, Perreault H. The usefulness of hydrazine derivatives for mass spectrometric analysis of carbohydrates. *Mass Spectrom Rev*. 2013;32:366-385. [Epub ahead of print].
229. Laughlin ST, Bertozzi CR. Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nat Protoc*. 2007;2:2930-2944.
230. Sletten EM, Bertozzi CR. From mechanism to mouse: a tale of two bioorthogonal reactions. *Acc Chem Res*. 2011;44:666-676.
231. Whiteaker JR, Lin C, Kennedy J, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat Biotechnol*. 2011;29:625-634.
232. Aebersold R. Constellations in a cellular universe. *Nature*. 2003;422:115-116.
233. Craig R, Cortens JP, Beavis RC. The use of proteotypic peptide libraries for protein identification. *Rapid Commun Mass Spectrom*. 2005;19:1844-1850.
234. Le Bihan T, Robinson MD, Stewart II, Figeys D. Definition and characterization of a "trypsinosome" from specific peptide characteristics by nano-HPLC-MS/MS and in silico analysis of complex protein mixtures. *J Proteome Res*. 2004;3:1138-1148.

References

1. Brooks JD. Translational genomics: the challenge of developing cancer biomarkers. *Genome Res*. 2012;22:183-187.
2. Ball P. DNA: Celebrate the unknowns. *Nature*. 2013;496:419-420.
3. Varki A. Omics: Account for the "dark matter" of biology. *Nature*. 2013;497:565.
4. Mischak H, Ioannidis JP, Argiles A, et al. Implementation of proteomic biomarkers: making it work. *Eur J Clin Invest*. 2012;42:1027-1036.
5. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003;422:198-207.
6. Check E. Proteomics and cancer: running before we can walk? *Nature*. 2004;429:496-497.
7. Master SR. Diagnostic proteomics: back to basics? *Clin Chem*. 2005;51:1333-1334.
8. Ransohoff DF. Lessons from controversy: ovarian cancer screening and serum proteomics. *J Natl Cancer Inst*. 2005;97:315-319.
9. Addona TA, Abbatiello SE, Schilling B, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat Biotechnol*. 2009;27:633-641.
10. Gloriam DE, Orchard S, Bertinetti D, et al. A community standard format for the representation of protein affinity reagents. *Mol Cell Proteomics*. 2010;9:1-10.
11. Paulovich AG, Billheimer D, Ham AJ, et al. Interlaboratory study characterizing a yeast performance standard for benchmarking LC-MS platform performance. *Mol Cell Proteomics*. 2010;9:242-254.
12. Rudnick PA, Clauser KR, Kilpatrick LE, et al. Performance metrics for liquid chromatography-tandem mass spectrometry systems in proteomics analyses. *Mol Cell Proteomics*. 2010;9:225-241.
13. Drake PM, Schilling B, Niles RK, et al. A lectin affinity workflow targeting glycosite-specific, cancer-related carbohydrate structures in trypsin-digested human plasma. *Anal Biochem*. 2011;408:71-85.
14. Drake PM, Schilling B, Niles RK, et al. Lectin chromatography/mass spectrometry discovery workflow identifies putative biomarkers of aggressive breast cancers. *J Proteome Res*. 2012;11:2508-2520.
15. Tabb DL, Vega-Montoto L, Rudnick PA, et al. Repeatability and reproducibility in proteomic identifications by liquid chromatography-tandem mass spectrometry. *J Proteome Res*. 2010;9:761-776.
16. Gaudet P, Argoud-Puy G, Cusin I, et al. neXtProt: organizing protein knowledge in the context of human proteome projects. *J Proteome Res*. 2013;12:293-298.
17. Hamacher M, Grottrup B, Eisenacher M, et al. Inter-lab proteomics: data mining in collaborative projects on the basis of the HUPO brain proteome project's pilot studies. *Methods Mol Biol*. 2011;696:235-246.
18. Legrain P, Aebersold R, Archakov A, et al. The human proteome project: current state and future direction. *Mol Cell Proteomics*. 2011.
19. Orchard S. Molecular interaction databases. *Proteomics*. 2012;12:1656-1662.
20. Stoevesandt O, Taussig MJ. European and international collaboration in affinity proteomics. *Nat Biotechnol*. 2012;29:511-514.
21. Lamond AI, Uhlen M, Horning S, et al. Advancing cell biology through proteomics in space and time (PROSPECTS). *Mol Cell Proteomics*. 2012;11:O112.017731.
22. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol*. 2006;24:971-983.
23. Cox J, Mann M. Is proteomics the new genomics? *Cell*. 2007;130:395-398.
24. Surinova S, Schiess R, Huttenhain R, Cerciello F, Wollscheid B, Aebersold R. On the development of plasma protein biomarkers. *J Proteome Res*. 2011;10:5-16.
25. Taylor CF, Hermjakob H, Julian Jr RK, Garavelli JS, Aebersold R, Apweiler R. The work of the Human Proteome Organisation's Proteomics Standards Initiative (HUPO PSI). *OMICS*. 2006;10:145-151.
26. Taylor CF, Paton NW, Lilley KS, et al. The minimum information about a proteomics experiment (MIAPE). *Nat Biotechnol*. 2007;25:887-893.
27. Rappsilber J, Mann M. What does it mean to identify a protein in proteomics? *Trends Biochem Sci*. 2002;27:74-78.
28. Kinsinger CR, Apffel J, Baker M, et al. Recommendations for mass spectrometry data quality metrics for open access data (corollary to the Amsterdam principles). *Proteomics Clin App*. 2011;5:580-589.
29. Tan HT, Lee YH, Chung MC. Cancer proteomics. *Mass Spectrom Rev*. 2012;31:583-605.
30. Rodriguez H, Snyder M, Uhlen M, et al. Recommendations from the 2008 International Summit on Proteomics Data Release and Sharing Policy: the Amsterdam principles. *J Proteome Res*. 2009;8:3689-3692.
31. Anderson JF, Siller E, Barral JM. Disorders of protein biogenesis and stability. *Protein Pept Lett*. 2011;18:110-121.
32. Copley SD. Moonlighting is mainstream: paradigm adjustment required. *Bioessays*. 2012;34:578-588.
33. Gidalevitz T, Prahlad V, Morimoto RI. The stress of protein misfolding: from single cells to multicellular organisms. *Cold Spring Harb Perspect Biol*. 2011;3:pii:a009704.
34. Nibbe RK, Chowdhury SA, Koyuturk M, Ewing R, Chance MR. Protein-protein interaction networks and subnetworks in the biology of disease. *Wiley Interdiscip Rev Syst Biol Med*. 2011;3:357-367.
35. Doucet A, Butler GS, Rodriguez D, Prudova A, Overall CM. Metadegradomics: toward in vivo quantitative degradomics of proteolytic post-translational modifications of the cancer proteome. *Mol Cell Proteomics*. 2008;7:1925-1951.
36. Meshorer E, Misteli T. Splicing misplaced. *Cell*. 2005;122:317-318.
37. Pakharukova NA, Pastushkova L, Moshkovskii SA, Larina IM. [Variability of healthy human proteome]. *Biomed Khim*. 2012;58:514-529.
38. Altshuler DM, Gibbs RA, Peltonen L, et al. Integrating common and rare genetic variation in diverse human populations. *Nature*. 2010;467:52-58.
39. Imanishi T, Itoh T, Suzuki Y, et al. Integrative annotation of 21,037 human genes validated by full-length cDNA clones. *PLoS Biol*. 2004;2:e162.
40. Kersey P, Bower L, Morris L, et al. Integr8 and Genome Reviews: integrated views of complete genomes and proteomes. *Nucleic Acids Res*. 2005;33:D297-D302.
41. Pruess M, Kersey P, Kulikova T, Apweiler R. Databases and resources for in silico proteome analysis. *Methods Biochem Anal*. 2006;49:397-414.
42. Omenn GS, States DJ, Adamski M, et al. Overview of the HUPO Plasma Proteome Project: results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database. *Proteomics*. 2005;5:3226-3245.
43. Zhang Q, Menon R, Deutsch EW, et al. A mouse plasma peptide atlas as a resource for disease proteomics. *Genome Biol*. 2008;9:R93.
44. Kamath KS, Vasavada MS, Srivastava S. Proteomic databases and tools to decipher post-translational modifications. *J Proteomics*. 2011;75:127-144.
45. Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW. Population proteomics: the concept, attributes, and potential for cancer biomarker research. *Mol Cell Proteomics*. 2006;5:1811-1818.
46. Asplund A, Edqvist PH, Schwenk JM, Ponten F. Antibodies for profiling the human proteome—The Human Protein Atlas as a resource for cancer research. *Proteomics*. 2012;12:2067-2077.
47. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248-1250.
48. Fry S, Afrough B, Leathem A, Dwek M. Lectin array-based strategies for identifying metastasis-associated changes in glycosylation. *Methods Mol Biol*. 2012;878:267-272.
49. Oliveira C, Teixeira JA, Domingues L. Recombinant lectins: an array of tailor-made glycan-interaction biosynthetic tools. *Crit Rev Biotechnol*. 2013;33:66-80.
50. Nagaraj N, Mann M. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. *J Proteome Res*. 2011;10:637-645.
51. Moreira JM, Cabezon T, Gromova I, et al. Tissue proteomics of the human mammary gland: towards an abridged definition of the molecular phenotypes underlying epithelial normalcy. *Mol Oncol*. 2010;4:539-561.
52. Ghosh SK, Yohannes E, Bebek G, et al. Proteomic and bioinformatic profile of primary human oral epithelial cells. *J Proteome Res*. 2012;11:5492-5502.
53. Zhou HJ, Liu YK, Chui JF, et al. A glycoproteome database of normal human liver tissue. *J Cancer Res Clin Oncol*. 2007;133:379-387.
54. Elortza F, Beretta L, Zhang X, He F, Mato JM. The 2011 Human Liver Proteome Project (HLPP) Workshop September 5, 2011, Geneva, Switzerland. *Proteomics*. 2012;12:5-8.

55. Grottrup B, Marcus K, Grinberg LT, Lee SK, Meyer HE, Park YM. Creating a human brain proteome atlas—14th HUPO BPP workshop September 20-21, 2010, Sydney, Australia. *Proteomics*. 2011;11:3269-3272.
56. Veenstra TD. Proteome analysis of posttranslational modifications. *Adv Protein Chem*. 2003;65:161-194.
57. Hancock WS, Wu SL, Shieh P. The challenges of developing a sound proteomics strategy. *Proteomics*. 2002;2:352-359.
58. McDonald WH, Yates 3rd JR. Shotgun proteomics and biomarker discovery. *Dis Markers*. 2002;18:99-105.
59. Wu CC, MacCoss MJ. Shotgun proteomics: tools for the analysis of complex biological systems. *Curr Opin Mol Ther*. 2002;4:242-250.
60. Schmidt A, Claassen M, Aebersold R. Directed mass spectrometry: towards hypothesis-driven proteomics. *Curr Opin Chem Biol*. 2009;13:510-517.
61. Kitteringham NR, Jenkins RE, Lane CS, Elliott VL, Park BK. Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877:1229-1239.
62. Shi T, Su D, Liu T, et al. Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics*. 2012;12:1074-1092.
63. Boja ES, Rodriguez H. Mass spectrometry-based targeted quantitative proteomics: achieving sensitive and reproducible detection of proteins. *Proteomics*. 2012;12:1093-1110.
64. Meng Z, Veenstra TD. Targeted mass spectrometry approaches for protein biomarker verification. *J Proteomics*. 2011;74:2650-2659.
65. Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*. 2004;3:235-244.
66. Jaffe JD, Keshishian H, Chang B, Addona TA, Gillette MA, Carr SA. Accurate inclusion mass screening: a bridge from unbiased discovery to targeted assay development for biomarker verification. *Mol Cell Proteomics*. 2008;7:1952-1962.
67. Smith RD, Anderson GA, Lipton MS, et al. An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics*. 2002;2:513-523.
68. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. *Science*. 1989;246:64-71.
69. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem*. 1988;60:2299-2301.
70. Catherman AD, Li M, Tran JC, et al. Top down proteomics of human membrane proteins from enriched mitochondrial fractions. *Anal Chem*. 2013;85:1880-1888.
71. Michalski A, Damoc E, Lange O, et al. Ultra high resolution linear ion trap Orbitrap mass spectrometer (Orbitrap Elite) facilitates top down LC MS/MS and versatile peptide fragmentation modes. *Mol Cell Proteomics*. 2012;11:O111.013698.
72. Young NL, Plazas-Mayorca MD, DiMaggio PA, et al. Collective mass spectrometry approaches reveal broad and combinatorial modification of high mobility group protein A1a. *J Am Soc Mass Spectrom*. 2010;21:960-970.
73. Frahm JL, Howard BE, Heber S, Muddiman DC. Accessible proteomics space and its implications for peak capacity for zero-, one- and two-dimensional separations coupled with FT-ICR and TOF mass spectrometry. *J Mass Spectrom*. 2006;41:281-288.
74. Sandhu C, Hewel JA, Badis G, et al. Evaluation of data-dependent versus targeted shotgun proteomic approaches for monitoring transcription factor expression in breast cancer. *J Proteome Res*. 2008;7:1529-1541.
75. Juhasz P, Lynch M, Sethuraman M, et al. Semi-targeted plasma proteomics discovery workflow utilizing two-stage protein depletion and off-line LC-MALDI MS/MS. *J Proteome Res*. 2011;10:34-45.
76. Savitski MM, Fischer F, Mathieson T, Sweetman G, Lang M, Bantscheff M. Targeted data acquisition for improved reproducibility and robustness of proteomic mass spectrometry assays. *J Am Soc Mass Spectrom*. 2010;21:1668-1679.
77. Wang N, Li L. Exploring the precursor ion exclusion feature of liquid chromatography–electrospray ionization quadrupole time-of-flight mass spectrometry for improving protein identification in shotgun proteome analysis. *Anal Chem*. 2008;80:4696-4710.
78. Yokosuka T, Yoshinari K, Kobayashi K, et al."Information-Based-Acquisition" (IBA) technique with an ion-trap/time-of-flight mass spectrometer for high-throughput and reliable protein profiling. *Rapid Commun Mass Spectrom*. 2006;20:2589-2595.
79. Zerck A, Nordhoff E, Resemann A, et al. An iterative strategy for precursor ion selection for LC-MS/MS based shotgun proteomics. *J Proteome Res*. 2009;8:3239-3251.
80. Graumann J, Scheltema RA, Zhang Y, Cox J, Mann M. A framework for intelligent data acquisition and real-time database searching for shotgun proteomics. *Mol Cell Proteomics*. 2012;11: M111.013185.
81. Liu H, Yang L, Khainovski N, et al. Automated iterative MS/MS acquisition: a tool for improving efficiency of protein identification using a LC-MALDI MS workflow. *Anal Chem*. 2011;83:6286-6293.
82. Nesvizhskii AI. Protein identification by tandem mass spectrometry and sequence database searching. *Methods Mol Biol*. 2007;367:87-119.
83. Bromenshenk JJ, Henderson CB, Wick CH, et al. Iridovirus and microsporidian linked to honey bee colony decline. *PLoS One*. 2010;5:e13181.
84. Knudsen GM, Chalkley RJ. The effect of using an inappropriate protein database for proteomic data analysis. *PLoS One*. 2011;6:e20873.
85. Albrethsen J. The first decade of MALDI protein profiling: a lesson in translational biomarker research. *J Proteomics*. 2011;74:765-773.
86. Kuwata H, Yip TT, Yip CL, Tomita M, Hutchens TW. Bactericidal domain of lactoferrin: detection, quantitation, and characterization of lactoferricin in serum by SELDI affinity mass spectrometry. *Biochem Biophys Res Commun*. 1998;245:764-773.
87. Merchant M, Weinberger SR. Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis*. 2000;21:1164-1177.
88. Szajli E, Feher T, Medzihradszky KF. Investigating the quantitative nature of MALDI-TOF MS. *Mol Cell Proteomics*. 2008;7:2410-2418.
89. Fung ET. A recipe for proteomics diagnostic test development: the OVA1 test, from biomarker discovery to FDA clearance. *Clin Chem*. 2010;56:327-329.
90. Exploring FDA-approved IVDMIAs; 2011. Retrieved from http://www.ivdtechnology.com/article/exploring-fda-approved-ivdmias.
91. Moore LE, Pfeiffer RM, Zhang Z, Lu KH, Fung ET, Bast Jr RC. Proteomic biomarkers in combination with CA 125 for detection of epithelial ovarian cancer using prediagnostic serum samples from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. *Cancer*. 2012;118:91-100.
92. Callesen AK, Mogensen O, Jensen AK, et al. Reproducibility of mass spectrometry based protein profiles for diagnosis of ovarian cancer across clinical studies: a systematic review. *J Proteomics*. 2012;75:2758-2772.
93. Purvine S, Eppel JT, Yi EC, Goodlett DR. Shotgun collision-induced dissociation of peptides using a time of flight mass analyzer. *Proteomics*. 2003;3:847-850.
94. Geromanos SJ, Vissers JP, Silva JC, et al. The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependent LC-MS/MS. *Proteomics*. 2009;9:1683-1695.
95. Silva JC, Gorenstein MV, Li GZ, Vissers JP, Geromanos SJ. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol Cell Proteomics*. 2006;5:144-156.
96. Gillet LC, Navarro P, Tate S, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics*. 2012;11:O111.016717.
97. Liu Y, Huttenhain R, Surinova S, et al. Quantitative measurements of N-linked glycoproteins in human plasma by SWATH-MS. *Proteomics*. 2013;13:1247-1256.
98. Horvatovich P, Hoekman B, Govorukhina N, Bischoff R. Multidimensional chromatography coupled to mass spectrometry in analysing complex proteomics samples. *J Sep Sci*. 2010;33:1421-1437.
99. Angel TE, Aryal UK, Hengel SM, et al. Mass spectrometry-based proteomics: existing capabilities and future directions. *Chem Soc Rev*. 2012;41:3912-3928.
100. Harvey SR, Macphee CE, Barran PE. Ion mobility mass spectrometry for peptide analysis. *Methods*. 2011;54:454-461.
101. Valentine SJ, Liu X, Plasencia MD, et al. Developing liquid chromatography ion mobility mass spectrometry techniques. *Expert Rev Proteomics*. 2005;2:553-565.
102. Kalnina Z, Zayakin P, Silina K, Line A. Alterations of pre-mRNA splicing in cancer. *Genes Chromosomes Cancer*. 2005;42:342-357.
103. Pajares MJ, Ezponda T, Catena R, Calvo A, Pio R, Montuenga LM. Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol*. 2007;8:349-357.

104. Omenn GS, Yocum AK, Menon R. Alternative splice variants, a new class of protein cancer biomarker candidates: findings in pancreatic cancer and breast cancer with systems biology implications. *Dis Markers*. 2010;28:241-251.
105. Kaida D, Schneider-Poetsch T, Yoshida M. Splicing in oncogenesis and tumor suppression. *Cancer Sci*. 2012;103:1611-1616.
106. Ferreira EN, Galante PA, Carraro DM, de Souza SJ. Alternative splicing: a bioinformatics perspective. *Mol Biosyst*. 2007;3:473-477.
107. Power KA, McRedmond JP, de Stefani A, Gallagher WM, Gaora PO. High-throughput proteomics detection of novel splice isoforms in human platelets. *PLoS One*. 2009;4:e5001.
108. Menon R, Omenn GS. Proteomic characterization of novel alternative splice variant proteins in human epidermal growth factor receptor 2/neu-induced breast cancers. *Cancer Res*. 2010;70:3440-3449.
109. Brosch M, Saunders GI, Frankish A, et al. Shotgun proteomics aids discovery of novel protein-coding genes, alternative splicing, and "resurrected" pseudogenes in the mouse genome. *Genome Res*. 2011;21:756-767.
110. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics*. 2006;5:573-588.
111. Qian WJ, Kaleta DT, Petritis BO, et al. Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol Cell Proteomics*. 2008;7:1963-1973.
112. Freeman WM, Lull ME, Guilford MT, Vrana KE. Depletion of abundant proteins from non-human primate serum for biomarker studies. *Proteomics*. 2006;6:3109-3113.
113. Gong Y, Li X, Yang B, et al. Different immunoaffinity fractionation strategies to characterize the human plasma proteome. *J Proteome Res*. 2006;5:1379-1387.
114. Polaskova V, Kapur A, Khan A, Molloy MP, Baker MS. High-abundance protein depletion: comparison of methods for human plasma biomarker discovery. *Electrophoresis*. 2010;31:471-482.
115. Yadav AK, Bhardwaj G, Basak T, et al. A systematic analysis of eluted fraction of plasma post immunoaffinity depletion: implications in biomarker discovery. *PLoS One*. 2011;6:e24442.
116. Whiteaker JR, Zhang H, Eng JK, et al. Head-to-head comparison of serum fractionation techniques. *J Proteome Res*. 2007;6:828-836.
117. Bellei E, Bergamini S, Monari E, et al. High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins. *Amino Acids*. 2011;40:145-156.
118. Camaggi CM, Zavatto E, Gramantieri L, et al. Serum albumin-bound proteomic signature for early detection and staging of hepatocarcinoma: sample variability and data classification. *Clin Chem Lab Med*. 2010;48:1319-1326.
119. Gundry RL, Fu Q, Jelinek CA, Van Eyk JE, Cotter RJ. Investigation of an albumin-enriched fraction of human serum and its albuminome. *Proteomics Clin App*. 2007;1:73-88.
120. Scumaci D, Gaspari M, Saccomanno M, et al. Assessment of an ad hoc procedure for isolation and characterization of human albuminome. *Anal Biochem*. 2011;418:161-163.
121. Koutroukides TA, Guest PC, Leweke FM, et al. Characterization of the human serum depletome by label-free shotgun proteomics. *J Sep Sci*. 2011;34:1621-1626.
122. Sennels L, Salek M, Lomas L, Boschetti E, Righetti PG, Rappsilber J. Proteomic analysis of human blood serum using peptide library beads. *J Proteome Res*. 2007;6:4055-4062.
123. Thulasiraman V, Lin S, Gheorghiu L, et al. Reduction of the concentration difference of proteins in biological liquids using a library of combinatorial ligands. *Electrophoresis*. 2005;26:3561-3571.
124. Roux-Dalvai F, Gonzalez de Peredo A, Simo C, et al. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol Cell Proteomics*. 2008;7:2254-2269.
125. Ernoult E, Bourreau A, Gamelin E, Guette C. A proteomic approach for plasma biomarker discovery with iTRAQ labelling and OFFGEL fractionation. *J Biomed Biotechnol*. 2010;2010:927917.
126. Colzani M, Waridel P, Laurent J, Faes E, Ruegg C, Quadroni M. Metabolic labeling and protein linearization technology allow the study of proteins secreted by cultured cells in serum-containing media. *J Proteome Res*. 2009;8:4779-4788.
127. Bandhakavi S, Van Riper SK, Tawfik PN, et al. Hexapeptide libraries for enhanced protein PTM identification and relative abundance profiling in whole human saliva. *J Proteome Res*. 2012;10:1052-1061.
128. Lorkova L, Pospisilova J, Lacheta J, et al. Decreased concentrations of retinol-binding protein 4 in sera of epithelial ovarian cancer patients: a potential biomarker identified by proteomics. *Oncol Rep*. 2012;27:318-324.
129. Monari E, Casali C, Cuoghi A, et al. Enriched sera protein profiling for detection of non-small cell lung cancer biomarkers. *Proteome Sci*. 2011;9:55.
130. Elliott MH, Smith DS, Parker CE, Borchers C. Current trends in quantitative proteomics. *J Mass Spectrom*. 2009;44:1637-1660.
131. Liang S, Xu Z, Xu X, Zhao X, Huang C, Wei Y. Quantitative proteomics for cancer biomarker discovery. *Comb Chem High Throughput Screen*. 2012;15:221-231.
132. Rajcevic U, Niclou SP, Jimenez CR. Proteomics strategies for target identification and biomarker discovery in cancer. *Front Biosci*. 2009;14:3292-3303.
133. Neilson KA, Ali NA, Muralidharan S, et al. Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics*. 2011;11:535-553.
134. Rebecchi KR, Wenke JL, Go EP, Desaire H. Label-free quantitation: a new glycoproteomics approach. *J Am Soc Mass Spectrom*. 2009;20:1048-1059.
135. Zhou W, Liotta LA, Petricoin EF. The spectra count label-free quantitation in cancer proteomics. *Cancer Genomics Proteomics*. 2012;9:135-142.
136. Ross PL, Huang YN, Marchese JN, et al. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics*. 2004;3:1154-1169.
137. Thompson A, Schafer J, Kuhn K, et al. Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem*. 2003;75:1895-1904.
138. Ong SE, Blagoev B, Kratchmarova I, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002;1:376-386.
139. Kashyap MK, Harsha HC, Renuse S, et al. SILAC-based quantitative proteomic approach to identify potential biomarkers from the esophageal squamous cell carcinoma secretome. *Cancer Biol Ther*. 2010;10:796-810.
140. Boersema PJ, Geiger T, Wisniewski JR, Mann M. Quantification of the N-glycosylated secretome by super-SILAC during breast cancer progression and in human blood samples. *Mol Cell Proteomics*. 2013;12:158-171.
141. Geiger T, Cox J, Ostasiewicz P, Wisniewski JR, Mann M. Super-SILAC mix for quantitative proteomics of human tumor tissue. *Nat Methods*. 2010;7:383-385.
142. Lund RR, Terp MG, Laenkholm AV, Jensen ON, Leth-Larsen R, Ditzel HJ. Quantitative proteomics of primary tumors with varying metastatic capabilities using stable isotope-labeled proteins of multiple histogenic origins. *Proteomics*. 2012;12:2139-2148.
143. Hakomori SI, Murakami WT. Glycolipids of hamster fibroblasts and derived malignant-transformed cell lines. *Proc Natl Acad Sci U S A*. 1968;59:254-261.
144. Meezan E, Wu HC, Black PH, Robbins PW. Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. II. Separation of glycoproteins and glycopeptides by sephadex chromatography. *Biochemistry*. 1969;8:2518-2524.
145. de Leoz ML, Young LJ, An HJ, et al. High-mannose glycans are elevated during breast cancer progression. *Mol Cell Proteomics*. 2011;10: M110.002717.
146. Comunale MA, Wang M, Rodemich-Betesh L, et al. Novel changes in glycosylation of serum Apo-J in patients with hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2011;20:1222-1229.
147. Ahn YH, Lee JY, Kim YS, Ko JH, Yoo JS. Quantitative analysis of an aberrant glycoform of TIMP1 from colon cancer serum by L-PHA-enrichment and SISCAPA with MRM mass spectrometry. *J Proteome Res*. 2009;8:4216-4224.
148. Li B, An HJ, Kirmiz C, Lebrilla CB, Lam KS, Miyamoto S. Glycoproteomic analyses of ovarian cancer cell lines and sera from ovarian cancer patients show distinct glycosylation changes in individual proteins. *J Proteome Res*. 2008;7:3776-3788.
149. Leiserowitz GS, Lebrilla C, Miyamoto S, et al. Glycomics analysis of serum: a potential new biomarker for ovarian cancer? *Int J Gynecol Cancer*. 2008;18:470-475.
150. Kyselova Z, Mechref Y, Kang P, et al. Breast cancer diagnosis and prognosis through quantitative measurements of serum glycan profiles. *Clin Chem*. 2008;54:1166-1175.
151. Kirmiz C, Li B, An HJ, et al. A serum glycomics approach to breast cancer biomarkers. *Mol Cell Proteomics*. 2007;6:43-55.

152. Ahn YH, Kim KH, Shin PM, Ji ES, Kim H, Yoo JS. Identification of low-abundance cancer biomarker candidate TIMP1 from serum with lectin fractionation and peptide affinity enrichment by ultrahigh-resolution mass spectrometry. *Anal Chem*. 2012;84:1425-1431.
153. Drake PM, Cho W, Li B, et al. Sweetening the pot: adding glycosylation to the biomarker discovery equation. *Clin Chem*. 2010;56:223-236.
154. Dube DH, Bertozzi CR. Glycans in cancer and inflammation–potential for therapeutics and diagnostics. *Nat Rev Drug Discov*. 2005;4:477-488.
155. Kim EH, Misek DE. Glycoproteomics-based identification of cancer biomarkers. *Int J Proteomics*. 2011;2011:601937. doi: 10.1155/2011/601937. Epub 2011 Sep 28.
156. Ruhaak LR, Miyamoto S, Lebrilla CB. Developments in the identification of glycan biomarkers for the detection of cancer. *Mol Cell Proteomics*. 2013;12:146-155.
157. Angata T, Fujinawa R, Kurimoto A, et al. Integrated approach toward the discovery of glyco-biomarkers of inflammation-related diseases. *Ann N Y Acad Sci*. 2012;1253:159-169.
158. Adamczyk B, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochim Biophys Acta*. 2012;1820:1347-1353.
159. Lee A, Chick JM, Kolarich D, et al. Liver membrane proteome glycosylation changes in mice bearing an extra-hepatic tumor. *Mol Cell Proteomics*. 2011;10: M900538MCP200. doi: 10.1074/mcp.M900538-MCP200. Epub 2010 Feb 18.
160. Balog CI, Stavenhagen K, Fung WL, et al. N-glycosylation of colorectal cancer tissues: a liquid chromatography and mass spectrometry-based investigation. *Mol Cell Proteomics*. 2012;11:571-585.
161. Hammarstrom S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol*. 1999;9:67-81.
162. van Gisbergen KP, Aarnoudse CA, Meijer GA, Geijtenbeek TB, van Kooyk Y. Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin. *Cancer Res*. 2005;65:5935-5944.
163. Jankovic MM, Milutinovic BS. Glycoforms of CA125 antigen as a possible cancer marker. *Cancer Biomark*. 2008;4:35-42.
164. Moss EL, Hollingworth J, Reynolds TM. The role of CA125 in clinical practice. *J Clin Pathol*. 2005;58:308-312.
165. Meany DL, Zhang Z, Sokoll LJ, Zhang H, Chan DW. Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. *J Proteome Res*. 2009;8:613-619.
166. Ohyama C, Hosono M, Nitta K, et al. Carbohydrate structure and differential binding of prostate specific antigen to *Maackia amurensis* lectin between prostate cancer and benign prostate hypertrophy. *Glycobiology*. 2004;14:671-679.
167. Vermassen T, Speeckaert MM, Lumen N, Rottey S, Delanghe JR. Glycosylation of prostate specific antigen and its potential diagnostic applications. *Clin Chim Acta*. 2012;413:1500-1505.
168. Narimatsu H, Iwasaki H, Nakayama F, et al. Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. *Cancer Res*. 1998;58:512-518.
169. Vestergaard EM, Hein HO, Meyer H, et al. Reference values and biological variation for tumor marker CA 19-9 in serum for different Lewis and secretor genotypes and evaluation of secretor and Lewis genotyping in a Caucasian population. *Clin Chem*. 1999;45:54-61.
170. Weiland F, Martin K, Oehler MK, Hoffmann P. Deciphering the molecular nature of ovarian cancer biomarker CA125. *Int J Mol Sci*. 2012;13:10568-10582.
171. Vanderschaeghe D, Festjens N, Delanghe J, Callewaert N. Glycome profiling using modern glycomics technology: technical aspects and applications. *Biol Chem*. 2010;391:149-161.
172. Mahal LK. Glycomics: towards bioinformatic approaches to understanding glycosylation. *Anticancer Agents Med Chem*. 2008;8:37-51.
173. Desaire H. Glycopeptide analysis: recent developments and applications. *Mol Cell Proteomics*. 2013;12:893-901.
174. Mechref Y, Hu Y, Garcia A, Hussein A. Identifying cancer biomarkers by mass spectrometry-based glycomics. *Electrophoresis*. 2012;33:1755-1767.
175. Pan S, Chen R, Aebersold R, Brentnall TA. Mass spectrometry based glycoproteomics—from a proteomics perspective. *Mol Cell Proteomics*. 2011;10: R110.003251.
176. Rakus JF, Mahal LK. New technologies for glycomic analysis: toward a systematic understanding of the glycome. *Annu Rev Anal Chem (Palo Alto Calif)*. 2011;4:367-392.
177. Kuzmanov U, Kosanam H, Diamandis EP. The sweet and sour of serological glycoprotein tumor biomarker quantification. *BMC Med*. 2013;11:31.
178. Wuhrer M. Glycomics using mass spectrometry. *Glycoconj J*. 2013;30:11-22.
179. Narimatsu H, Sawaki H, Kuno A, Kaji H, Ito H, Ikehara Y. A strategy for discovery of cancer glyco-biomarkers in serum using newly developed technologies for glycoproteomics. *FEBS J*. 2010;277:95-105.
180. Patwa T, Li C, Simeone DM, Lubman DM. Glycoprotein analysis using protein microarrays and mass spectrometry. *Mass Spectrom Rev*. 2010;29:830-844.
181. Zhang Y, Yin H, Lu H. Recent progress in quantitative glycoproteomics. *Glycoconj J*. 2012;29:249-258.
182. Pasing Y, Sickmann A, Lewandrowski U. N-glycoproteomics: mass spectrometry-based glycosylation site annotation. *Biol Chem*. 2012;393:249-258.
183. Saba J, Dutta S, Hemenway E, Viner R. Increasing the productivity of glycopeptides analysis by using higher-energy collision dissociation-accurate mass-product-dependent electron transfer dissociation. *Int J Proteomics*. 2012;2012:560391.
184. Hanisch FG. O-glycoproteomics: site-specific O-glycoprotein analysis by CID/ETD electrospray ionization tandem mass spectrometry and top-down glycoprotein sequencing by in-source decay MALDI mass spectrometry. *Methods Mol Biol*. 2012;842:179-189.
185. Wang H, Wong CH, Chin A, et al. Integrated mass spectrometry-based analysis of plasma glycoproteins and their glycan modifications. *Nat Protoc*. 2011;6:253-269.
186. Darula Z, Sherman J, Medzihradszky KF. How to dig deeper? Improved enrichment methods for mucin core-1 type glycopeptides. *Mol Cell Proteomics*. 2012;11: O111.016774.
187. von der Lieth CW, Bohne-Lang A, Lohmann KK, Frank M. Bioinformatics for glycomics: status, methods, requirements and perspectives. *Brief Bioinform*. 2004;5:164-178.
188. von der Lieth CW, Lutteke T, Frank M. The role of informatics in glycobiology research with special emphasis on automatic interpretation of MS spectra. *Biochim Biophys Acta*. 2006;1760:568-577.
189. Apte A, Meitei NS. Bioinformatics in glycomics: glycan characterization with mass spectrometric data using SimGlycan. *Methods Mol Biol*. 2010;600:269-281.
190. Maley F, Trimble RB, Tarentino AL, Plummer Jr TH. Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem*. 1989;180:195-204.
191. Kaji H, Isobe T. Stable isotope labeling of N-glycosylated peptides by enzymatic deglycosylation for mass spectrometry-based glycoproteomics. *Methods Mol Biol*. 2013;951:217-227.
192. Wada Y, Dell A, Haslam SM, et al. Comparison of methods for profiling O-glycosylation: Human Proteome Organisation Human Disease Glycomics/Proteome Initiative multi-institutional study of IgA1. *Mol Cell Proteomics*. 2010;9:719-727.
193. Hanisch FG. Chemical de-O-glycosylation of glycoproteins for applications in LC-based proteomics. *Methods Mol Biol*. 2011;753:323-333.
194. Jung K, Cho W, Regnier FE. Glycoproteomics of plasma based on narrow selectivity lectin affinity chromatography. *J Proteome Res*. 2009;8:643-650.
195. Abbott KL, Pierce JM. Lectin-based glycoproteomic techniques for the enrichment and identification of potential biomarkers. *Methods Enzymol*. 2010;480:461-476.
196. Fanayan S, Hincapie M, Hancock WS. Using lectins to harvest the plasma/serum glycoproteome. *Electrophoresis*. 2012;33:1746-1754.
197. Bond MR, Kohler JJ. Chemical methods for glycoprotein discovery. *Curr Opin Chem Biol*. 2007;11:52-58.
198. Geng M, Zhang X, Bina M, Regnier F. Proteomics of glycoproteins based on affinity selection of glycopeptides from tryptic digests. *J Chromatogr B Biomed Sci Appl*. 2001;752:293-306.
199. Comunale MA, Wang M, Hafner J, et al. Identification and development of fucosylated glycoproteins as biomarkers of primary hepatocellular carcinoma. *J Proteome Res*. 2009;8:595-602.
200. Xiong L, Andrews D, Regnier F. Comparative proteomics of glycoproteins based on lectin selection and isotope coding. *J Proteome Res*. 2003;2:618-625.

201. Durham M, Regnier FE. Targeted glycoproteomics: serial lectin affinity chromatography in the selection of O-glycosylation sites on proteins from the human blood proteome. *J Chromatogr A*. 2006;1132:165-173.
202. Calvano CD, Zambonin CG, Jensen ON. Assessment of lectin and HILIC based enrichment protocols for characterization of serum glycoproteins by mass spectrometry. *J Proteomics*. 2008;71:304-317.
203. Heo SH, Lee SJ, Ryoo HM, Park JY, Cho JY. Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. *Proteomics*. 2007;7:4292-4302.
204. Yang Z, Harris LE, Palmer-Toy DE, Hancock WS. Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. *Clin Chem*. 2006;52:1897-1905.
205. Plavina T, Wakshull E, Hancock WS, Hincapie M. Combination of abundant protein depletion and multi-lectin affinity chromatography (M-LAC) for plasma protein biomarker discovery. *J Proteome Res*. 2007;6:662-671.
206. Kullolli M, Hancock WS, Hincapie M. Preparation of a high-performance multi-lectin affinity chromatography (HP-M-LAC) adsorbent for the analysis of human plasma glycoproteins. *J Sep Sci*. 2008;31:2733-2739.
207. Drake P, Schilling B, Gibson B, Fisher S. Elucidation of N-glycosites within human plasma glycoproteins for cancer biomarker discovery. *Methods Mol Biol*. 2013;951:307-322.
208. Yang Z, Hancock WS. Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column. *J Chromatogr A*. 2004;1053:79-88.
209. Abbott KL, Aoki K, Lim JM, et al. Targeted glycoproteomic identification of biomarkers for human breast carcinoma. *J Proteome Res*. 2008;7:1470-1480.
210. Cho W, Jung K, Regnier FE. Use of glycan targeting antibodies to identify cancer-associated glycoproteins in plasma of breast cancer patients. *Anal Chem*. 2008;80:5286-5292.
211. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat Biotechnol*. 2003;21:660-666.
212. Tian Y, Zhou Y, Elliott S, Aebersold R, Zhang H. Solid-phase extraction of N-linked glycopeptides. *Nat Protoc*. 2007;2:334-339.
213. Sparbier K, Asperger A, Resemann A, et al. Analysis of glycoproteins in human serum by means of glycospecific magnetic bead separation and LC-MALDI-TOF/TOF analysis with automated glycopeptide detection. *J Biomol Tech*. 2007;18:252-258.
214. Zhang H, Loriaux P, Eng J, et al. UniPep—a database for human N-linked glycosites: a resource for biomarker discovery. *Genome Biol*. 2006;7:R73.
215. An HJ, Miyamoto S, Lancaster KS, et al. Profiling of glycans in serum for the discovery of potential biomarkers for ovarian cancer. *J Proteome Res*. 2006;5:1626-1635.
216. de Leoz ML, An HJ, Kronewitter S, et al. Glycomic approach for potential biomarkers on prostate cancer: profiling of N-linked glycans in human sera and pRNS cell lines. *Dis Markers*. 2008;25:243-258.
217. Bereman MS, Williams TI, Muddiman DC. Development of a nanoLC LTQ orbitrap mass spectrometric method for profiling glycans derived from plasma from healthy, benign tumor control, and epithelial ovarian cancer patients. *Anal Chem*. 2009;81:1130-1136.
218. Bereman MS, Young DD, Deiters A, Muddiman DC. Development of a robust and high throughput method for profiling N-linked glycans derived from plasma glycoproteins by NanoLC-FTICR mass spectrometry. *J Proteome Res*. 2009;8:3764-3770.
219. Tang Z, Varghese RS, Bekesova S, et al. Identification of N-glycan serum markers associated with hepatocellular carcinoma from mass spectrometry data. *J Proteome Res*. 2010;9:104-112.
220. Hua S, An HJ, Ozcan S, et al. Comprehensive native glycan profiling with isomer separation and quantitation for the discovery of cancer biomarkers. *Analyst*. 2011;136:3663-3671.
221. Alley Jr WR, Vasseur JA, Goetz JA, et al. N-linked glycan structures and their expressions change in the blood sera of ovarian cancer patients. *J Proteome Res*. 2012;11:2282-2300.
222. Bones J, Mittermayr S, O'Donoghue N, Guttman A, Rudd PM. Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. *Anal Chem*. 2010;82:10208-10215.
223. Hua S, Lebrilla C, An HJ. Application of nano-LC-based glycomics towards biomarker discovery. *Bioanalysis*. 2011;3:2573-2585.
224. Isailovic D, Plasencia MD, Gaye MM, et al. Delineating diseases by IMS-MS profiling of serum N-linked glycans. *J Proteome Res*. 2012;11:576-585.
225. Aldredge D, An HJ, Tang N, Waddell K, Lebrilla CB. Annotation of a serum N-glycan library for rapid identification of structures. *J Proteome Res*. 2012;11:1958-1968.
226. Wuhrer M, de Boer AR, Deelder AM. Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry. *Mass Spectrom Rev*. 2009;28:192-206.
227. Hua S, Williams CC, Dimapasoc LM, et al. Isomer-specific chromatographic profiling yields highly sensitive and specific potential N-glycan biomarkers for epithelial ovarian cancer. *J Chromatogr A*. 2013;1279:58-67.
228. Lattová E, Perreault H. The usefulness of hydrazine derivatives for mass spectrometric analysis of carbohydrates. *Mass Spectrom Rev*. 2013;32:366-385. [Epub ahead of print].
229. Laughlin ST, Bertozzi CR. Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nat Protoc*. 2007;2:2930-2944.
230. Sletten EM, Bertozzi CR. From mechanism to mouse: a tale of two bioorthogonal reactions. *Acc Chem Res*. 2011;44:666-676.
231. Whiteaker JR, Lin C, Kennedy J, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. *Nat Biotechnol*. 2011;29:625-634.
232. Aebersold R. Constellations in a cellular universe. *Nature*. 2003;422:115-116.
233. Craig R, Cortens JP, Beavis RC. The use of proteotypic peptide libraries for protein identification. *Rapid Commun Mass Spectrom*. 2005;19:1844-1850.
234. Le Bihan T, Robinson MD, Stewart II, Figeys D. Definition and characterization of a "trypsinosome" from specific peptide characteristics by nano-HPLC-MS/MS and in silico analysis of complex protein mixtures. *J Proteome Res*. 2004;3:1138-1148.

Elaine R. Mardis

23

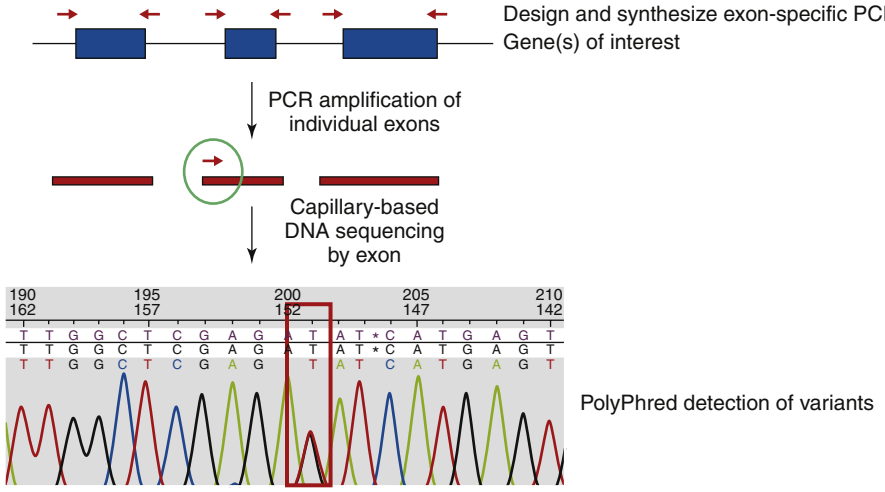
The Technology of Analyzing Nucleic Acids in Cancer

Introduction to Next-Generation Sequencing

Current DNA sequencing methods differ dramatically from a mere 7 years ago, when next-generation sequencing (NGS) instrumentation was first introduced.[1] Indeed, the science of DNA sequencing is only 35 years old, and its companion discipline known as *genomics* has been revolutionized by the advent of NGS instrumentation and its application to myriad biological questions. Whereas conventional DNA cloning and sequencing approaches, largely based on the initial descriptions by Fred Sanger and colleagues,[2-5] provided key reference genome sequences for model organisms (*Caenorhabditis elegans*, *Escherichia coli*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Mus musculus*) and for humans,[6] next-generation instruments and associated analytical efforts have truly revolutionized the nature of biological inquiry. This fact is nowhere more evident than in the study of cancer, first proposed by Bovery as a disease whose origins lay in profound alterations of the nuclear DNA, remarkably before DNA was found to be the hereditary material and instruction set for the organism. His hypothesis about cancer as a disease of the genome was first supported by microscopic observations of reoccurring chromosomal translocations in leukemic cells.[7-10] Later, the fusion proteins resulting from these translocations were proven necessary and sufficient to induce the developmental arrest and proliferation of leukemic cells. Hence, genomic alterations were a precursor to cancer's development.[11-14]

Having the reference human genome in hand, the combination of polymerase chain reaction (PCR) and Sanger sequencing by fluorescent capillary methods provided the first nucleotide-level evidence that point mutations in tyrosine kinase genes also were associated with carcinogenesis.[15,16] Thus, DNA sequencing was shown to provide a higher resolution "microscope" with which to catalog specific genes that were commonly mutated in cancerous cell genomes. These efforts also established the rationale that genes found recurrently mutated in different cancer samples and ultimately across different types of tumors were likely drivers of oncogenesis. Although increasing the scale of this approach was attainable, there were known limitations to PCR- and Sanger-based approaches (Figure 23-1). On a practical level, PCR had many sources of failure, including the inability to design primers that bind with fidelity in repetitive sequences or exclude pseudogenes or distinguish specific genes in gene families, as well as amplification failure when primer annealing failed due either to DNA polymorphisms or to structural alterations that changed or removed one or both binding sites or to poor amplicon yield from high G+C content (common in the first exons of genes). Furthermore, data generation for large numbers of genes required a significant amount of DNA to be obtained from each tumor. In addition, although large-scale automation could be employed to provide throughput and reproducibility, scaling successfully to address all human genes in large numbers of samples was cost prohibitive. Beyond practical considerations, the approach provided no information about DNA rearrangements or copy number alterations and overall no appreciation of reoccurring events outside the genes.

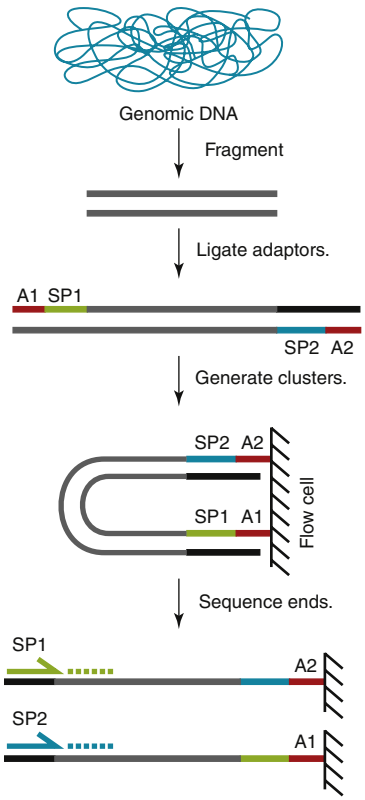
The advent of NGS instruments addressed many of these limitations by providing several advantages in terms of throughput and cost.[1] Although the technical nuances of the various NGS instruments differ, they generally share the same principles (Figure 23-2). These include (1) a simplicity of library construction that requires comparatively little DNA versus PCR-based methods, (2) an enzymatic amplification of each library fragment to produce sufficient signal during the sequencing reaction, and (3) a stepwise sequencing reaction that detects the signal from each nucleotide incorporation reaction of each amplified fragment population before moving to the next reaction. This en masse sequencing approach is why NGS is often referred to as "massively parallel." Indeed, dramatic increases in scale and speed, and decreases in cost, of



Pros:
Low computational overhead for data analysis
Scalable to medium throughput

Cons:
Primer design failures
Amplification failures
DNA consuming
Expensive
No structural variation or copy number information

Figure 23-1 PCR and capillary sequencing of exons A generalized workflow is shown for the PCR-based amplification of specific exons in the human genome, their sequencing, and separation of the nucleotide sequence by a fluorescent capillary sequencing approach. Nucleotide changes can be identified by the appropriate software, such as PolyPhred, which was widely used for analysis of capillary data. A single base substitution mutation is shown in the trace data example. Pros and cons of the approach are listed and described in the text.



Pros:
Rapid inexpensive data generation of whole genome data
Comprehensive analysis possible for nearly all somatic events
Low input DNA amounts possible
Scalable

Cons:
Large data volumes are computationally challenging
Short read lengths

Figure 23-2 Next-generation sequencing (NGS) of whole genomes A generalized workflow for the production of whole-genome sequencing data by next-generation or massively parallel sequencing is shown. Several pros and cons of this approach are listed and described in the text. Computational identification of the genome-wide differences between tumor and normal genomes requires highly specialized pipelines for each variant type (point mutation, copy number alteration, insertion/deletion variant, structural variant).

data generation by NGS have occurred in a very short time. The size of NGS datasets, especially from whole-genome sequencing (discussed later), coupled with relatively short read lengths compared to Sanger sequencing, have required substantial investments in the development of computer algorithms, computing/IT infrastructure, and computational biology expertise to successfully interpret the data.[17] These computational efforts have been further taxed by continued improvements in NGS data quality (error rates), increasing read lengths, and the ability to generate sequencing data from both ends of each library fragment (typically referred to as "paired end" sequencing) because of the need for new algorithm and data analysis pipeline development as well as refinements to existing computational pipelines. The bottom line is

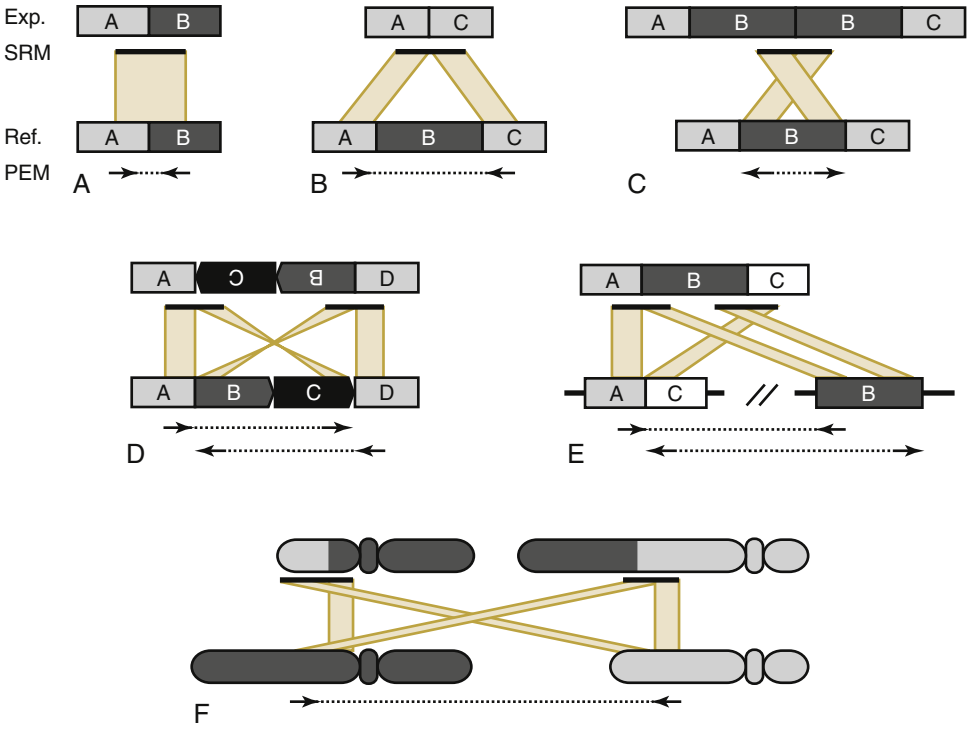


Figure 23-3 Read placement distance and orientation is indicative of structural variation of various types In each figure, the paired end mapping (PEM) orientation and distance on the reference genome (Ref) of the NGS data is shown relative to the short read mapping (SRM) of the experimental data (Exp). **(A)** The anticipated read mapping distance and orientation are shown. **(B)** A deletion in the experimental genome. **(C)** An insertion. **(D)** An inversion. **(E)** A complex rearrangement. **(F)** A translocation. *With permission from Quinlan AR, Hall IM. Characterizing complex structural variation in germline and somatic genomes.* Trends Genet. *2012;28:43-53.*

that the first step in interpreting short read data is computational alignment to a reference genome such as the Human Reference Genome.[18,19] Alignment maps each read or read pair to its origin in the genome and then assigns a quality score to the mapping position that can be interpreted for its certainty of correct placement. Subsequent analytical approaches further interpret the total read alignment (also called "coverage") in a variety of ways to identify single-nucleotide variants (SNVs), small insertion or deletion events that involve one or a few bases ("indels"), and structural variants (large insertion or deletion events, chromosomal inversions, or translocations–see Figure 23-3). Although read length and data quality improvements have overall expanded the utility of NGS for biological experimentation and decreased the cost of data generation by a trajectory that exceeds Moore's law, the computational requirements of NGS analysis have remained largely unchanged.[20] Many unique aspects, discussed later, further complicate sequencing and analysis from cancer samples. In spite of the obstacles, our understanding of the genomic landscape of cancer has changed remarkably in just a few short years.

Challenges to NGS Analysis of Cancer Nucleic Acids

The search for somatic variation in cancer DNA and RNA has a distinct advantage over other complex diseases: The exact comparison of tumor to normal nucleic acids within an individual patient distinctly identifies those alterations that are tumor unique. Furthermore, there are increasing amounts of data from various projects that have begun using NGS methods to catalog large numbers of cancer cases across different tumor types (ICGC [icgc.org], TCGA [cancergenome.nih.gov], PCGP [www.pediatriccancergenomeproject.org][21] that can be used to inform individual analyses about previously described alterations. In spite of these decided advantages, there are several significant challenges that confound experimental design and analytical approaches in cancer genomics studies. Several examples of these challenges are described next, along with the ways researchers attempt to overcome them, where applicable.

Tumor Cellularity

Cancerous cells in solid tumors do not exist in isolation in the body. Rather, they are always in close proximity to normal cells of various types, including stromal cells, immune cells, and components known as the *extracellular matrix* (ECM). The proportion of tumor cells can be estimated by an experienced pathologist examining the tumor section under hematoxylin and eosin staining, and this estimate is expressed as a "percent tumor nuclei" or "percent tumor cellularity" value. As a result of the association of tumor and normal cells, an isolate of DNA or RNA derived from a solid cancer sample will contain both tumor and normal cells unless a specific procedure such as flow cytometry or laser capture microdissection

(LCM) is used first to significantly enrich the percentage of tumor cells in the isolate. Also, certain tumor types, such as those from prostate or pancreas, are more prone to low tumor cellularity. Based on the pathology estimate, decisions in sequencing must be made in the context of tumor cellularity percentages. Namely, if the tumor cellularity is below 60%, the decision must be made either to enrich the tumor by flow cytometry (more common for blood cancers such as lymphoma or leukemia) or by LCM (used for solid tumors), or to try oversampling the tumor NGS library (increased sequencing coverage) by an amount commensurate with the tumor cellularity estimate. Although sorting or LCM seems the most obvious choice, one limitation of either approach is that significantly reduced yields of DNA or RNA will be obtained. Unless specialized procedures are in hand, the low yield may limit the ability to derive high-quality data from such samples. By contrast, oversampling may be effective for DNA sequencing but will be more expensive to generate and will require adjustment of variant calling parameters, or use of a more sensitive variant caller, to effectively identify somatic variants. Oversampling for RNA-seq from a sample with low tumor cellularity is generally not advised, as the tumor transcripts will be too difficult to discern from those of the normal cells unless LCM or sorting is first used to separate the tumor cells from the adjacent normal/nonmalignant cells.

Heterogeneity (Regional versus Genotypic)

Heterogeneity is a fundamental aspect of cancer cells found within the same tumor of which there are two types, regional and genotypic. Regional heterogeneity reflects the differences that emerge in solid tumors as they grow and progress. It refers to the different regions present in a tumor mass, such as areas of necrosis or areas of invasion (of surrounding normal tissue). Genotypic heterogeneity reflects the fact that cancer cells evolve during the process of tumor progression, so that not all tumor cells share the same somatic genotype. In genotypic heterogeneity, the use of NGS has demonstrated that by comparing the genomes from progression samples (a de novo leukemia compared to its relapse) using high-depth sequencing of somatic mutations, an initiating or "founder" clone can be identified that contains the core mutational load that initiates tumor growth as well as more advanced clones that combine newer mutations with those in the founder clone.[22] One shared aspect of regional and genotypic heterogeneity is that as a tumor mass increases in size, both are more likely to occur in that areas of regional heterogeneity are likely to have genotypic heterogeneity. There are so far only two studies to examine this at the DNA level; one study of two advanced-stage renal cell carcinomas that exhibited extreme genotypic heterogeneity[23] and one study of five early-stage (2/3) breast cancers that showed little to no genotypic heterogeneity[24] when sampled and studied at multiple sites.

Ploidy and Copy Number Alterations in DNA

Altered numbers of chromosomes (more or fewer than 2) have been widely observed in cancer cells, likely reflecting errors in chromosomal segregation that occur during rapid division and growth cycles. Observing ploidy alterations requires cytogenetic examination of the tumor cells in metaphase, which may or may not be part of the pathology-based diagnosis for the patient sample. Alternatively, ploidy alterations and large chromosomal arm or subarm amplifications and deletions (somatic copy number alterations or SCNAs) can be inferred from signal strength–based analysis of genotyping array data.[25,26] Ploidy increases and arm or subarm amplifications are important in DNA sequencing of the tumor because these regions will contribute more DNA to the library, and hence more reads will result than for the diploid (or haploid) regions of the genome. Thus increased coverage must be obtained for the tumor library to compensate for amplified regions or ploidy-altered chromosomes so the coverage of diploid genomic regions is sufficient for variant detection. Careful analysis of aligned reads in copy number–altered regions can provide exquisite resolution of the genes involved and of the relative timing of somatic mutation and copy number alteration when both occur in the same locus.[27]

FFPE Preservation and Nucleic Acid Integrity

Most pathology assays used in cancer diagnosis and characterization require stability of proteins and cellular structure. Hence, fixation in formalin and embedding in paraffin have been the standard pathology preparation methods for more than 100 years. As this practice is unlikely to change in the near future, and because so many clinically valuable specimens already have been preserved by this method, the study of formalin-fixed, paraffin-embedded (FFPE)-preserved nucleic acid isolates by NGS methods is increasing. The chemical reaction between formaldehyde, proteins, and nucleic acids leads to crosslinking proteins and nucleic acids, and ultimately the DNA/RNA backbone breaks because of the presence of abasic lesions.[28] This is a random interaction, and DNA/RNA fragmentation increases with longer exposure of nucleic acids to formalin and over time of storage. Therefore, the older a tumor FFPE block, the more likely to be advanced the degradation of the nucleic acid components. Nonetheless, careful examination of the nucleic acid integrity will identify those samples suitable for library construction for DNA or RNA, based on the average size

and distribution of degraded nucleic acid isolated from the sample. In DNA isolates, the average fragment size should be 300 bp or greater or a suitable NGS library is unlikely to result. In RNA, the 28S and 18S rRNA peaks should be visible by gel electrophoresis, with an RNA integrity number (RIN) of at least 5.

Applications of NGS to Study and Analyze Nucleic Acids

The genomic DNA isolated from cancer cell nuclei can be studied in a variety of ways, several of which are profiled here. Because cancer develops from alterations of the nuclear genome that are distinct from the germline genome, an inherent and powerful comparison can be obtained by studying the paired tumor and normal genomic DNA from individual cancer patients. In discovery efforts, large numbers of such cases can be studied to add information about the frequency of different types of somatic alterations and the genes whose protein products will be altered as a result. Higher level analyses of the pathways affected by somatic alterations in DNA can further inform the resulting tumor biology. Studies of RNA by NGS methods have deepened our understanding of the numerous types of RNAs, their membership, and how they are altered in the course of carcinogenesis, although not all alterations are comprehensible in the biological context. The latter reflects our ignorance of the many roles these molecules play in cellular biology, emphasizing the need for functional studies as a follow-on to NGS-mediated discovery efforts.

Whole-Genome Sequencing

The most comprehensive approach to identifying the somatic alterations present in cancer genomics is obtained by whole-genome sequencing (WGS) of the tumor and normal DNAs. In this approach (see Figure 23-2), the isolated high-molecular-weight genomic DNA from each tissue is fragmented by the application of high-frequency sound waves or other physical shearing methods and then enzymatically treated to blunt the fragment ends that result. Finally, short synthetic adapters are added to make a whole-genome library. After limited PCR amplification by primers that correspond to the forward and reverse adapters, a gel-based sizing allows specific size fractions to be isolated (two to four insert sizes are typical to enhance library diversity and genome representation). The more precise the size fraction, the more precisely structural variants can be identified by virtue of their relative position once mapped to the reference genome (see Figure 23-3). Libraries are then quantitated, diluted to the appropriate concentration, and amplified in situ to produce collections of fragments, each of which originated from a single library fragment. Thus the data generated from WGS are "digital" in nature and can be interpreted later in this context, to provide highly precise information about chromosomal amplification and deletion events genome-wide, and the relative frequency of mutations in the tumor cell genomes sampled by DNA isolation.[22] Read pair data are then generated from tumor and normal libraries to a minimum depth of 30-fold, allowing for a mapping rate of around 85% of read pairs; this equates roughly to 120 Gbp of data per genome. Following data generation, the signals obtained from the stepwise sequencing process are interpreted by instrument-specific software, culled for low-quality sequences, paired, and provided to the mapping algorithm for alignment to the Human Reference Genome as outlined earlier. Alignment is done for tumor-specific reads and for normal-specific reads separately. Variants are identified and then compared to one another.[29] There are many specialized algorithms that have been specifically developed to evaluate the somatic variants that are carried by the cancer cell genomes decoded by whole genome sequencing data. Depending upon the algorithm type, one can identify somatic single nucleotide variants (SNVs), focused insertion and deletion events of one to several nucleotides (in/dels), and larger, structural events such as translocations, inversions, deletions, and amplifications. Loss of heterozygosity (LOH) is a common somatic genome event, and there also are algorithms to identify stretches of LOH along chromosomes. Each algorithm has an associated false positive rate, so secondary validation of putative somatic variants is the best practice. The identification of structural variants is particularly prone to a high false positive rate due to the difficulty of identifying these regions, as illustrated in Figure 23-3. Here, the distance and orientation of read pair mapping to the Human Genome Reference for multiple unique read pairs is required to identify a structural event, as indicated in the figure. By using the read pairs that identify the event, and a short read assembly algorithm, one can reassemble the structural variant event to nucleotide resolution. Finally, one makes "sense" of the variants identified genome-wide by annotation, effectively overlaying our current understanding of genes, regulatory regions, and other identified features that help define the tumor-unique profile of genomic alterations.

As sequencing costs have dropped and instrument throughput has increased, the amount of read data and hence the coverage of the tumor genome has increased. This increase has occurred for several reasons: notably, the confidence of detecting somatic variations typically increases with increasing coverage. Furthermore, the heterogeneous nature of cancer cell genomes means that increased coverage

provides enhanced characterization of the mutational spectrum within the cells. Tumor progression is at its essence an evolutionary process in which new mutations arise from the fundamental tumor genome (often referred to as the "founder clone") and expand into new subpopulations of cells.[22,30] Thus, the higher the tumor genome coverage, the more likely it is that subpopulations can be identified.

Exome Sequencing

Although WGS data are straightforward to produce and provide comprehensive genome-wide information about somatic alterations, their production remains expensive, and they are difficult to accurately interpret. Much like the early PCR-directed methods used to characterize cancer somatic mutations, technology development efforts in NGS have resulted in an application typically referred to as "hybrid capture" to selectively isolate regions of the genome followed by NGS.[31-33] Sequence-based comparison of the isolated regions between tumor and normal generate specific information about somatic and germline SNVs and indels. Hybrid capture protocols combine the whole-genome library fragments from tumor and normal with a collection of specific probe sequences designed to capture, by hybridization, those fragments in the population that contain the same loci the probes represent. Because capture probes carry covalently attached biotin moieties, the probe:library fragment hybrids can be selectively removed from solution by association with streptavidin-coated magnetic particles and the application of a magnet. Unhybridized fragments are removed with the supernatant, and a secondary wash eliminates many (but not all) spurious hybridization events (typically referred to as "off-target effects"). The resulting captured fragments are eluted from the beads by denaturation, quantitated, and sequenced to about 100-fold average depth. In one commonly used version of hybrid capture, probes representing nearly all of the annotated exons in the human genome (the "exome") permit selective capture of these exons so they can be sequenced, compared, and annotated with respect to the somatic alterations identified. Exome capture reagents are available from commercial manufacturers, and the associated methods can be automated readily to provide a very high throughput of exome capture reactions, suitable for large-scale cancer discovery. As an alternative, custom capture probe sets can be designed and manufactured by one of several commercial suppliers to selectively isolate genes/loci of interest and characterize their mutational status in a large number of cancer cases or as a diagnostic reagent to assess specific mutational hotspots. This approach also can be used to select loci from a whole-genome library that carry putative variants, as a means of validation that mutations indeed exist.[22] Hybrid capture becomes of limited utility when the target loci (also referred to as "regions of interest" or ROIs) to be isolated fall below a combined length of approximately 500 kbp, mainly because the amount of off-target hybridization increases as the target space decreases. Because off-target captured fragments contribute to the overall fragment pool that is recovered for sequencing, the amount of sequence data actually mapping to the loci of interest decreases steadily to the point that data generation becomes too expensive to obtain the necessary coverage in desired targets. Below an ROI of about 500 kbp, either PCR and amplicon pooling or multiplex PCR are typically used.

DNA Methylation

One predominant mechanism of transcriptional control in cells is the covalent modification of the cytosine bases in DNA by methyl groups and their derivatives. Understanding the ways that methylation status changes in tumor cells can provide insights into changes in gene expression patterns, as well as new prognostic markers if sufficient clinical data and samples exist. This type of analysis requires comparator normal methylation data, ideally obtained from adjacent nonmalignant tissue genomic DNA isolates. Correlative analyses then can link DNA methylation changes to gene expression changes, providing insights into tumor biology that cannot be obtained by directly sequencing genomic DNA. Several approaches to identifying methylated cytosines in genomic DNA use chemical modification, antibody-based recognition of methylC, or comparative restriction enzyme digestion patterns from exposure to a methylation-sensitive versus non–methylation-sensitive isoschizomer. However, the most widely used approach is bisulfite modification.[34,35] In bisulfite modification, native genomic DNA is treated with sodium bisulfite to convert unmethylated cytosines to uracils (see Figure 23-4). When copied by a restriction enzyme, each unmethylated C will represent as a C to T transition, whereas methylated C residues are untouched and incorporate a G during copying. In the pre-NGS era, regions upstream of genes of interest that were activated or silenced by methylation changes were evaluated by comparing bisulfite treatment plus PCR to PCR alone between tumor and adjacent non-malignant (normal) DNA. In the era of NGS, whole genomic DNA of tumor and adjacent normal tissues are treated with bisulfite after library construction (the adapters are methylated to prevent their conversion) and then processed and sequenced as described earlier.[36] The resulting bisulfite converted reads are aligned in silico to a "bisulfite converted" genome in order to identify unmethylated (and by inference, methylated) C residues (Figure 23-4).

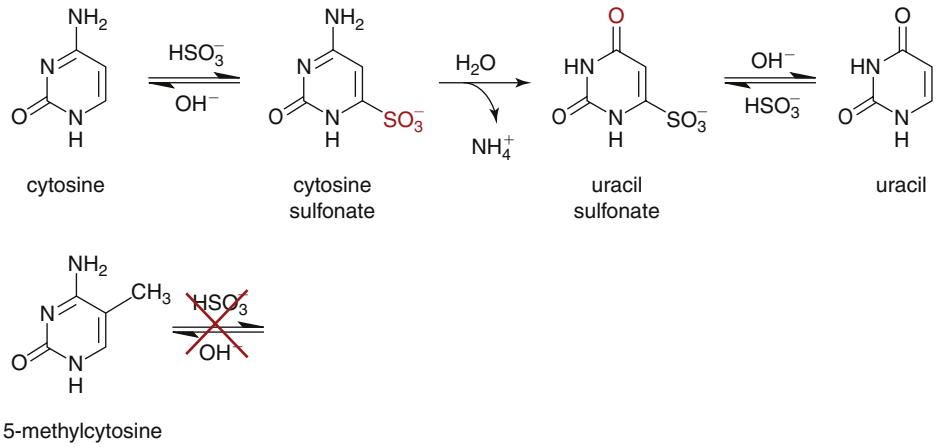


FIGURE 23-4 CHEMICAL CONVERSION OF UNMETHYLATED CYTOSINE RESIDUES BY BISULFITE The upper panel indicates the series of reactions that occur between unmethylated cytosine residues and the bisulfite reagent to sulfonate cytosine, eliminate ammonia to generate uracil sulfonate, and then eliminate bisulfite to generate uracil. In the lower panel, methylated cytosines do not participate in the reaction with bisulfite.

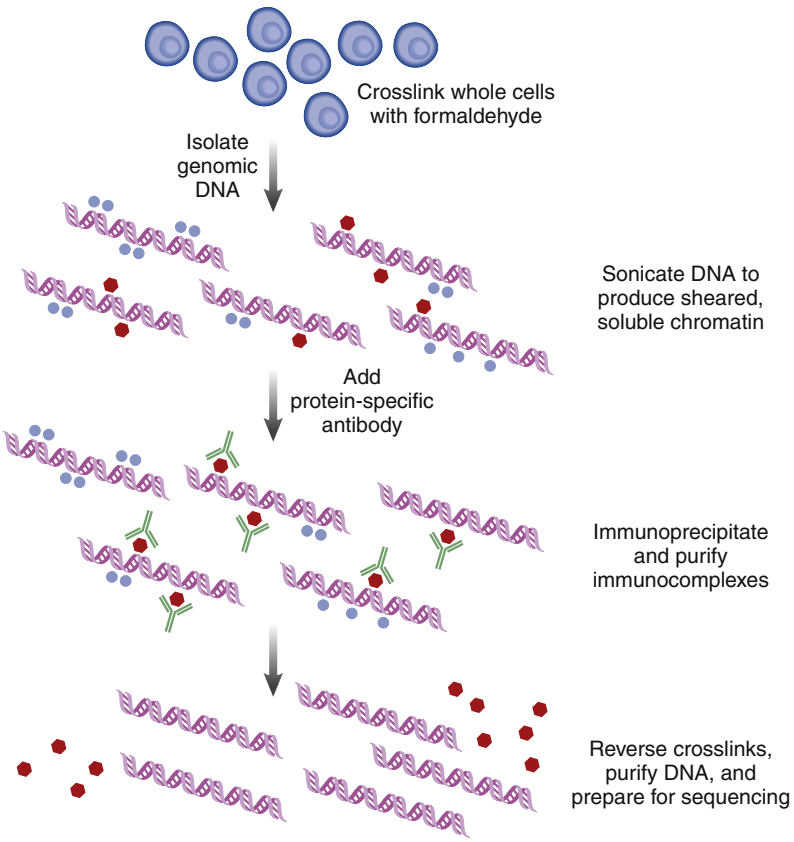


FIGURE 23-5 BASIC PRINCIPLES OF CHROMATIN IMMUNOPRECIPITATION (ChIP) After crosslinking DNA to proteins by treating live cells with formaldehyde, the DNA is isolated and then sheared to a uniform length distribution. By mixing the sheared DNA with a specific antibody to the protein of interest, crosslinked fragments can be bound and then isolated by immunoprecipitation. Following this step, reversing the crosslinking allows the DNA fragments to be reclaimed. The resulting low yield of DNA is converted to a next-generation sequencing (NGS) library and sequenced. Alignment to the reference genome and peak finding can identify those regions of the genome that are bound by the protein of interest.

Chromatin Immunoprecipitation

Genomic DNA contains myriad regulatory sites that provide the binding sequences recognized by the cell's transcriptional regulation machinery. In addition, complexing of the genomic DNA with histones provides a fundamental mechanism for permitting access to regions of the DNA so transcription can occur. Changes to binding sites due either to mutation or amplification/deletion can destroy protein binding and effectively silence the gene. Similarly, rearrangements of bound histones can make available or eliminate genes from transcriptional copying. One approach to evaluate these changes is broadly termed *chromatin immunoprecipitation*, or ChIP (see Figure 23-5). ChIP is based on two fundamental approaches: (1) DNA and protein in close physical proximity can be bound reversibly by the introduction of formalin to growing cell cultures, and (2) the DNA:protein complexes can be precipitated from solution by an antibody-mediated immunoprecipitation step. Before NGS methods, the genomic

region of interest was assayed from these immunoprecipitated DNA fragment populations by quantitative polymerase chain reaction (qPCR), allowing a selective look at protein binding status. In the NGS era, however, sequencing of the collective ChIP fraction can identify protein-bound regions.[37,38] These are challenging libraries to produce because of the low yield of DNA from the immunoprecipitation step. Once sequencing data are obtained, the reads are analyzed by first mapping onto the reference genome and then detecting the peaks that indicate factor- or histone-bound loci, as appropriate. The accuracy and sensitivity of these methods largely depend on the quality of the antibody used to effect immunoprecipitation, and on attaining enough coverage that peaks can be distinguished from noise.

Sequencing Messenger RNA (mRNA)

The pursuit to characterize RNA expression as a biomarker of prognosis, metastasis, therapeutic options, and other clinical metrics lies at the essence of cancer genomics. In particular, before sequencing was transformed by next-generation instrumentation, microarrays served as a mechanism to characterize tumor-specific gene expression in a reasonably comprehensive and measurable way. However, relative to microarrays, sequencing of coding RNAs (called "RNA-seq") provides data suitable for addressing a multitude of RNA-specific questions that one might use to characterize a tumor. For example, the digital nature of sequencing means that gene expression levels are quantifiable rather than relative, a concept first introduced by Wold and colleagues, who defined the metric known as FPKM (fragments per kilobase of transcript per million mapped reads) to express the normalized value of expression for each transcript detected.[39] Beyond gene expression levels, there are myriad levels of complexity that can be mined from RNA-seq datasets, including information about splice isoforms, allele-specific expression, chimeric or fusion transcripts, and RNA editing.[40,41] Much like ChIP-seq data, described earlier, these data are without context for an individual sample, unless a matched nonmalignant adjacent normal tissue is similarly prepared, sequenced, and evaluated. At present, there is a dearth of knowledge from RNA-seq analysis of normal human tissues to provide even a plausible baseline for these studies as an alternative to the adjacent nonmalignant tissue comparator. Integrating data from DNA and RNA sequencing can be incredibly powerful in the context of cancer genomics, such as DNA amplification leading to increased RNA expression levels for the genes lying in the amplified region (ERBB2 in breast cancer) or chromosomal translocation leading to a fusion gene transcript that drives oncogenesis (t15;17 and PML-RARα, t9:21 and BRC-ABL).

There are myriad approaches to producing RNA-seq data from tumor RNA isolates, depending on the yield obtained. Ideally, polyA selection is used for abundant total RNA samples (more than 2 μg) to reduce highly expressed rRNA transcripts, but these yields are not guaranteed with samples from clinical procedures such as fine-needle aspirates or LCM. In this regard, RNA-seq has benefitted from previous microarray studies because enzymatic RNA amplification is widely accepted to represent the RNA expression levels of the native sample. Amplification can be further refined by specialized approaches to RNA that accurately reflect the strandedness of the transcript or adjust for the fragmentation of RNA that is derived from FFPE preserved tissues. There are several methods that subsequently decrease the levels of abundant rRNA transcripts when polyA purification cannot be used, including selective amplification of polyA, use of abundance-based hybridization kinetics and selective double-strand hybrid depletion, or selective depletion using rRNA-targeted locked nucleic acids (LNAs).

Sequencing Noncoding RNAs

In addition to the transcripts from protein-coding genes, many classes of noncoding RNAs (ncRNAs) have been described and have been determined to be expressed at altered levels in cancer cells.[42,43] As for messenger RNAs (mRNAs), the digital nature of NGS can provide exquisite quantitation of expression levels while being comprehensive across a wide dynamic range of expression. Highly specialized library construction techniques for ncRNAs depend largely on a clever approach to selecting the ncRNAs from total RNA isolates and then performing a sizing step that isolates the specific population of interest. Obviously, the sequencing parameters of NGS instruments should be altered to specifically reflect the corresponding sizes of ncRNAs being studied and the types of analyses planned for the resulting data.

Conclusions

As illustrated by this overview, the impact of NGS and associated methods on our ability to characterize the nucleic acids involved in cancer, and hence to generate comprehensive hypotheses about tumor biology, has evolved rapidly. These methods and associated analyses continue to develop and are now coalescing toward diagnostic assays that provide an individualized focus for precise prognostic and therapeutic determinations. Over time, the cancer genomics revolution, fueled by NGS innovation, will profoundly affect the outcomes of cancer patients worldwide.

Key References

1. Mardis ER. A decade's perspective on DNA sequencing technology. *Nature*. 2011;470:198-203.
5. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74:5463-5467.
15. Sjoblom T, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
19. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2010;26:589-595.
22. Ding L, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506-510.
36. Lister R, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462:315-322.
38. Garber M, et al. A high-throughput chromatin immunoprecipitation approach reveals principles of dynamic gene regulation in mammals. *Mol Cell*. 2012;47:810-822.
40. Garber M, Grabherr MG, Guttman M, Trapnell C. Computational methods for transcriptome annotation and quantification using RNA-seq. *Nat Methods*. 2011;8:469-477.

This page intentionally left blank

References

1. Mardis ER. A decade's perspective on DNA sequencing technology. *Nature*. 2011;470:198-203.
2. Sanger F, et al. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature*. 1977;265:687-695.
3. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol*. 1975;94:441-448.
4. Sanger F, Coulson AR, Barrell BG, Smith AJ, Roe BA. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J Mol Biol*. 1980;143:161-178.
5. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977;74:5463-5467.
6. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. *Nature*. 2004;431:931-945.
7. Rowley JD, Golomb HM, Dougherty C. 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia. *Lancet*. 1977;1:549-550.
8. Rowley JD. The cytogenetics of acute leukaemia. *Clin Haematol*. 1978;7:385-406.
9. Rowley JD. Chromosome abnormalities in leukemia. *Haematol Blood Transfus*. 1979;23:43-52.
10. Rowley JD. Ph1-positive leukaemia, including chronic myelogenous leukaemia. *Clin Haematol*. 1980;9:55-86.
11. Rowley JD. Identification of the constant chromosome regions involved in human hematologic malignant disease. *Science*. 1982;216:749-751.
12. Rowley JD. Biological implications of consistent chromosome rearrangements in leukemia and lymphoma. *Cancer Res*. 1984;44:3159-3168.
13. Rowley JD. The Philadelphia chromosome translocation. A paradigm for understanding leukemia. *Cancer*. 1990;65:2178-2184.
14. Rowley JD. The role of chromosome translocations in leukemogenesis. *Semin Hematol*. 1999;36:59-72.
15. Sjoblom T, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
16. Pao W, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004;101:13306-13311.
17. Mardis ER, Wilson RK. Cancer genome sequencing: a review. *Hum Mol Genet*. 2009;18:R163-R168.
18. Li H, Ruan J, Durbin R. Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Res*. 2008;18:1851-1858.
19. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2010;26:589-595.
20. Schuster SC, et al. Complete Khoisan and Bantu genomes from southern Africa. *Nature*. 2010;463:943-947.
21. Hudson TJ, et al. International network of cancer genome projects. *Nature*. 2010;464:993-998.
22. Ding L, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506-510.
23. Gerlinger M, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
24. Ellis MJ, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature*. 2012;486:353-360.
25. Zhang Q, et al. CMDS: a population-based method for identifying recurrent DNA copy number aberrations in cancer from high-resolution data. *Bioinformatics*. 2010;26:464-469.
26. Beroukhim R, et al. Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. *Proc Natl Acad Sci U S A*. 2007;104:20007-20012.
27. Nik-Zainal S, et al. The life history of 21 breast cancers. *Cell*. 2012;149:994-1007.
28. Magana-Schwencke N, Ekert B, Moustacchi E. Biochemical analysis of damage induced in yeast by formaldehyde. I. Induction of single-strand breaks in DNA and their repair. *Mutat Res*. 1978;50:181-193.
29. Larson DE, et al. SomaticSniper: identification of somatic point mutations in whole genome sequencing data. *Bioinformatics*. 2012;28:311-317.
30. Yachida S, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*. 2010;467:1114-1117.
31. Albert TJ, et al. Direct selection of human genomic loci by microarray hybridization. *Nat Methods*. 2007;4:903-905.
32. Hodges E, et al. Genome-wide in situ exon capture for selective resequencing. *Nat Genet*. 2007;39:1522-1527.
33. Gnirke A, et al. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotechnol*. 2009;27:182-189.
34. Harris RA, et al. Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. *Nat Biotechnol*. 2010;28:1097-1105.
35. Frommer M, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A*. 1992;89:1827-1831.
36. Lister R, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462:315-322.
37. Goren A, et al. Chromatin profiling by directly sequencing small quantities of immunoprecipitated DNA. *Nat Methods*. 2010;7:47-49.
38. Garber M, et al. A high-throughput chromatin immunoprecipitation approach reveals principles of dynamic gene regulation in mammals. *Mol Cell*. 2012;47:810-822.
39. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. 2008;5:621-628.
40. Garber M, Grabherr MG, Guttman M, Trapnell C. Computational methods for transcriptome annotation and quantification using RNA-seq. *Nat Methods*. 2011;8:469-477.
41. Hawkins RD, Hon GC, Ren B. Next-generation genomics: an integrative approach. *Nat Rev Genet*. 2010;11:476-486.
42. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med*. 2012;4:143-159.
43. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. *Cancer Discov*. 2011;1:391-407.

Paul T. Spellman, Josh Stuart, and Joe W. Gray

24

Understanding and Using Information about Cancer Genomes

High-resolution genome analysis techniques are now being used in international cancer genome analysis efforts to catalog aberrations driving the pathophysiology of nearly all major cancer types. The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) project and the International Cancer Genome Consortium (ICGC, http://www.icgc.org/)[1] represent the largest of these. The TCGA project is assessing aberrations in 500 to 1000 tumors from each of about 20 major human cancer types and together the TCGA and ICGC are currently committed to the analysis of ~47 separate tumor lineages. Results for glioblastoma,[2] serous ovarian cancer,[3] colon cancer,[4] squamous cell lung cancer,[5] and breast cancer[6] are already available from the TCGA project, and other technology centers around the world have contributed studies of cancers of the breast,[7-10] pancreas,[11] prostate,[12] lung,[13] and kidney[14] and of melanoma,[15] myeloma,[16] and AML.[17] The overall intent of these international genomics efforts is to provide a knowledge base of cancer genome landscapes that can be used to develop more effective cancer management strategies—for example, by enabling early detection of lethal or recurrent cancers, diagnosing patients with low malignancy potential tumors who may be spared from aggressive treatment, identifying novel therapeutic targets, and assigning therapies to the patients in which they are most likely to be effective. We summarize in this chapter general and specific aspects of human cancers that are emerging from international genomics efforts, describe computational and experimental efforts to identify aberrations that contribute to aspects of human cancer pathophysiology, and illustrate how genomic information is being used in aspects of cancer management.

The Emerging Cancer Genome Landscape

Genome aberrations found to be important in human cancers are illustrated in Figure 24-1[18] and include (1) somatic changes in copy number that increase or decrease the levels of important coding and noncoding RNA transcripts, (2) somatic mutations that alter gene expression, protein structure, or protein stability and/or change the way transcripts are spliced, (3) structural changes that affect transcript levels by altering gene-promoter associations or create new fusion genes, and (4) epigenomic events that alter transcription levels of diverse signaling pathways and that enable rapid emergence of therapeutically resistant subpopulations. Table 24-1 shows the number of gene aberrations catalogued in the Cancer Gene Census maintained by the Sanger Institute that have been implicated in one or more human cancer types (see http://www.sanger.ac.uk/genetics/CGP/Census). These aberrations have been implicated in deregulation of pathways that influence all aspects of cancer progression including invasion, immortalization, DNA replication and repair, proliferation, apoptosis, angiogenesis, motility, and adhesion. However, the 487 genes implicated in the Cancer Gene Census are likely to be only a small fraction of the aberrant genetic events that play important roles in the form and function of human cancers. These genes have been selected primarily because they were implicated in model system studies or because they occur frequently in one or more cancer subtypes. However, recent genomic studies demonstrate that hundreds to thousands of genes may be affected by somatic mutations and epigenomic modifications in an individual tumor. These comprise a mix of "driver" aberrations and a usually larger number of "passenger" aberrations. Driver aberrations are genomic or epigenomic events that are selected during tumor progression because they alter one or more aspects of cell and tissue physiology to allow cancer initiation, progression, and/or dissemination. Table 24-2, for example, illustrates several genes that might contribute to the deregulation of the cancer hallmarks designated by Weinberg and Hanahan.[19] Some of these aberrations have been targeted by therapeutics, and others have been shown to influence clinical behavior, including response to targeted and nontargeted therapies.[19] Passenger aberrations do not contribute to cancer pathophysiology but arise by chance

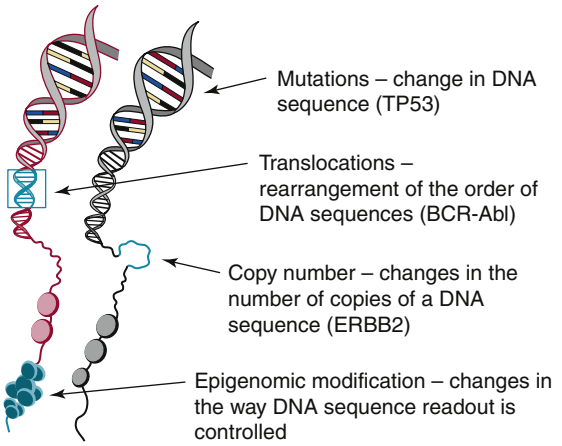


Figure 24-1 Schematic illustrations of the types of genome aberrations found in human cancers.[18]

Table 24-1 Cancer Gene Census Summary

Aberration Type	Number of Aberrations	Examples of Prominent Affected Genes
Amplification	16	ERBB2, EGFR, MYCN, MDM2, CCND1
Frameshift mutation	100	APC, RB1, ATM, MLH1, NF1
Germline mutation	76	BRCA1/2, TP53, ERCC2, RB1, VHL
Missense mutation	141	ARID1A, ATM, PIK3CA, IDH1, KRAS
Nonsense mutation	92	CDKN2A, FANCA, PTCH, PTEN
Other mutation	26	BRAF, PDGFRA, PIK3R1, SOCS1
Splicing mutation	63	GATA3, MEN1, MSH2, TSC1
Translocation	326	ABL1, ALK, BCL2, TMPRSS2, MYC

For more details see http://www.sanger.ac.uk/genetics/CGP/Census.

Table 24-2 Candidate Cancer Hallmark–Associated Aberrant Genes

Cancer Hallmark	Aberrant Gene
Resisting cell death	BCL2, BAX, FAS
Genome instability and mutation	TP53, BRCA1/2, MLH1
Inducing angiogenesis	CCK2R
Activating invasion and metastasis	ADAMTSL4, ADAMTS3
Tumor-promoting inflammation	IL32
Enabling replicative immortality	TERT
Avoiding immune destruction	HLA loci, TAP1/2, B2M
Evading growth suppressors	RB1, CCND1, CDKN2A
Sustaining proliferative signaling	KRAS, ERBB2, MYC
Deregulating cellular energetics	PIK3CA, PTEN

during progression in a genomically unstable tumor and are carried along because they exist in cells carrying driver aberrations. Driver aberrations are identified either because they occur frequently in tumor subpopulations or because they have been identified as contributing to aspects of cancer pathophysiology in laboratory models of cancer—for example, cultured cancer cells or genetically manipulated nonmalignant cells, or cancer cells grown as xenografts in animals—or have been demonstrated to influence aspects of cancer in genetically engineered living organisms. Several general cancer genomic observations from international cancer genomics projects are summarized in the following paragraphs.

One important observation from many genomic studies is the existence of recurrent molecular features that allow cancers that occur in specific anatomic regions to be organized into subtypes. The subtypes likely arise in distinct cell types within each tissue and are different diseases that differ in clinical outcome and/or response to therapy. Early genomic studies relied on expression patterns for cancer subtype definition, but current strategies use multiple data types (e.g., genome copy number, mutation, and expression) for subtype definition. Interestingly, epithelial and mesenchymal subtypes appear to be present in tumors that are of epithelial origin. The mesenchymal-like cancers tend to be more rapidly proliferating and motile and associated with reduced survival duration. Some tumor types show remarkably high transcriptional similarity, for example, in triple-negative breast cancer and high-grade serous ovarian cancers.[6] Many genomic aberrations also appear in multiple tumor subtypes. Some of the most common aberrations observed in multiple tumor types include amplifications of MYC and EGFR, deletion of CDKN2A and PTEN, and mutation of TP53 and PIK3CA. For a more comprehensive assessment, Kim and colleagues summarize recurrent genome copy number aberrations in 8000 cancers.[20] Efforts are now under way to combine data types (e.g., expression, genome copy number, and mutations) to increase the number of subtypes in order to increase the precision with which patients can be stratified according to outcome and/or therapeutic response.[21] Of course, this divides cancers into increasingly smaller subpopulations, so very large numbers of samples are needed to establish subtype differences in treatment response or overall outcome.

The number of aberrations that are present in an individual tumor can be remarkably high. The somatic mutation rate in human cancers varies between cancer types from about 0.1 to 10 mutations per megabase,[22,23] but individual tumors may carry as few as a hundred to more than a million somatic aberrations. High genomic instability occurs because of loss of telomere function during progression in the absence of telomerase,[24,25] diminished DNA repair capacity resulting from genomic and epigenomic deregulation of DNA repair pathways,[26] increased damage resulting from oncogene-induced

oxidative stress,[27] and toxic environmental exposures.[28,29] In some cases, the exact DNA sequence change in a mutation reflects the type of agent that causes the cancer—for example, mutations in sun-related cancers show CC to TT mutations caused by UV-induced cytosine dimers, whereas smoking-induced cancers in the lung are characterized by G→T transversions caused by the polycyclic aromatic hydrocarbons in tobacco smoke.[30,31] Ultimately, the functions and/or expression levels of hundreds to thousands of genes may be altered in an individual tumor. An unknown number of these will be drivers. Among these, some will have a strong, possibly dominant influence on an individual tumor, whereas others may have a more modest or near-negligible impact. So far, most attention in the field has focused on the strong drivers. However, it seems likely that the ensemble of aberrations will have to be taken into account in explaining the overall behavior of an individual tumor, which is addressed in a later section.

The same drivers of genome instability that enable tumor development also operate during tumor progression. As a result, individual tumors become increasingly heterogeneous as distinct clonal populations within the tumor evolve in diverse microenvironments, producing highly branched lineages. For example, events that enable metastasis may occur late during the genetic evolution,[32] whereas mutation of TP53, a key player in genome stability, can be an early event.[33] These instabilities and the resultant intratumor heterogeneity in an individual tumor are likely responsible for the rapid evolution of therapeutic resistance. This heterogeneity complicates clinical decision making because the importance of a low-frequency but actionable aberration remains unclear. One possible way forward is to focus treatment on aberrations that occur early during tumor development. The order in which aberrations occur can be inferred by examining a tissue at various stages of disease progression[34] by serial sampling of clinical tissue from individual patients,[18] by computational methods that examine mutation frequency,[35-37] or in some cases by analysis of the interactions between mutations and copy-number abnormalities.[33]

Functional Assessment of Cancer Genomes

Transforming cancer genomic data into interpretable knowledge consists of finding the parts and learning how they work together to enable aspects of cancer pathophysiology. Hypothesis-driven research has gone quite far in this process, but full understanding will require systematic analysis, both computational and experimental, of the aberrations that occur within a tumor genome.

Computational Approaches

Computational strategies to identify candidate driver aberrations begin with the cataloging of all aberrations and then move to the selection of high-priority candidate drivers.

Cataloging Approaches

Identification of genes that enable aspects of cancer pathophysiology (driver genes) is complicated by the high genomic heterogeneity within and between tumors. Nearly all cancer genomes analyzed to date appear to have at least one driving oncogenic point mutation, and the vast majority show copy number changes over both large chromosomal segments and smaller, more targeted regions of the genome. The evidence for structural rearrangements being a primary cause in most tumor types is less clear, but diseases including many leukemias, lymphomas, sarcomas, and prostate cancers all incontrovertibly show that rearrangements can be critical (http://atlasgeneticsoncology.org). Changes to chromatin state also are partly responsible for many cancers.[38-40] Over the past 20 years a number of technologies (predominantly microarray based) have been successfully used to catalog cancer genome aberrations, but nearly all efforts now depend on nucleic acid sequencing technology (Mardis, chapter on "The Technology of Analyzing Nucleic Acids").

Point mutations are identified by aligning DNA sequences obtained from cancer samples to normal genomes using tools such as BWA.[41] The requirement for the normal genome sequence is paramount because of private single-nucleotide polymorphisms (SNPs) that occur about once every 100,000 base pairs,[42] a rate that is about 10 times higher than the mutation rate in most epithelial tumors and 100 times higher than the rate of mutations in childhood cancers such as neuroblastoma.[3,6,22] Read depth and read quality are critical factors in determining how well mutations can be called within each patient's cancer genome. Read quality is the error rate per thousand base pairs of sequence. High quality is usually defined as having fewer than 1 error per 1000 bases of sequence. Read depth (the number of times a position in the genome has been sequenced) for high-quality bases then governs both the false-positive rate caused by sequencing errors and misidentifying private variants as mutations and false negatives caused by not generating sufficient data to observe mutations reliably. The greater the depth, the more confident mutation calls will be. Typically, 30× coverage of the normal genome and 40× to 80× coverage of the tumor produces high-quality results. Increasing read depth is needed for analysis of samples in which the tumor fraction is low because the presence of normal DNA reads dilutes the aberrant reads. Mutation detection is further complicated by intratumor heterogeneity that causes some aberrations to be present in only a

small fraction of the tumor cells. Many groups find value in exome sequencing—that is, targeting the small fraction of the genome that is coding, at even deeper levels (for example, 150×). Verifying the sensitivity of mutation calling remains difficult because there are no good true mutation standards.

Detection of insertions and deletions (indels) remains challenging. In principle, the same sequence coverage necessary to find point mutations can be used to identify indels. Unfortunately, the algorithmic methods for indel identification are much more computationally intense.[43] No good estimates exist on how well indel detection software works because of the lack of gold standards against which to measure algorithm performance. In general, indel detection is even more difficult than evaluating the substitution mutations.

Copy number and structural aberrations are identified using a combination of microarray and sequencing approaches. Microarrays and whole-genome shotgun sequencing are capable of identifying changes in DNA copy number that are as small as 1000 base pairs in length. This resolution is sufficiently good that nearly all gene-level aberrations can be detected. Microarray approaches look for differential signal gains from the hybridization, whereas DNA sequences detect changes in read depth. Direct sequencing of genomic DNA represents the most direct way to identify the breakpoints for structural rearrangements, but the methodology is challenging, requiring a high-coverage, high-quality DNA sequence. Often, structural rearrangements cannot be detected with the standard technologies because the sequencing approaches used cannot span the length of repetitive sequences in the human genome. Once a whole-genome shotgun sequence is generated, methods such as BreakDancer[44] and Delly[45] can be used to find the chromosome junctions. Other structural aberration detection technologies are emerging, so it is likely that we will be able to identify the majority of structural breakpoints in the near future.

Detection of promoter methylation is usually accomplished using microarray technologies. Microarrays that can measure methylation at more than 485,000 sites are now commonly used by groups such as TCGA.[7] In principle, DNA sequencing can be used for this purpose, but this is currently economically impractical, with costs 10 to 50 times greater than for microarray approaches. In addition, sequencing approaches currently require unreasonably large quantities of tumor DNA.

RNAseq is now the standard for measuring gene expression. RNA is depleted of ribosomal RNA (rRNA) by either polyA+ selection or any number of rRNA depletion steps and fragmented before complementary DNA (cDNA) production. Short cDNA fragments are sequenced and mapped to the human genome reference. Algorithms to estimate which transcripts are being produced and their relative abundances[46] are used to interpret the fragment data. One strength of RNAseq analysis is that it does not require that the transcriptome be known, and thus it has enabled the study of noncoding RNAs, including lincRNAs and, with adapted protocols, miRNAs.[47,48] RNAseq methods are still being refined, with improvements in molecular and algorithmic approaches regularly being developed.

Integrating Information

A central challenge in cancer genomics today is in distinguishing the causal components of disease from the effects of the disease, or even more importantly from the random aberrations that occur during progression and are carried along by chance association with driver mutations. Suites of tools have been developed to answer these key questions.

The major focus of efforts such as TCGA and ICGC has been to identify the recurrently mutated genes in specific cancer types. For example, in serous ovarian cancer 95% of all tumors have point mutations in *TP53*. Statistics are not needed for the average scientist to decide that *TP53* is a critical gene. In most cases, however, the process for deciding if a gene is recurrently mutated in a specific tumor type is much more complicated, even after one has identified the mutations. First, not all genes are of the same length; longer genes should have more mutations by chance if mutations are equally likely at each position. Failure to control for gene size often leads to the identification of genes encoding long proteins such as Titin, whose coding sequence is 100 times longer than that of the average human gene. Second, mutations within a tumor type are not evenly split among all possibilities. For example, tumors caused by UV light will show high rates of C→T mutations in general, especially at CC dinucleotides. Further, we now know that mutations are not randomly distributed over the genome. For example, regions of the genome near late replication forks can have mutation rates 10 times higher than the average rate. Without accounting for this, many genes will be identified as showing more mutations than expected by chance when in fact they do not.[49] Identifying driver genes based on patterns of recurrence is partly about understanding the mutagenic processes as a whole and performing appropriate statistical tests to incorporate them.[5,6]

Many genes have hotspots where mutations occur preferentially. For example, mutations in the *HRAS* gene have a bias to alter the 12th amino acid to valine from glycine. When these events occur repeatedly, similar statistics for overall mutation rate can be used, but instead constrained for a specific event. Thus, with far fewer examples, a specific gene mutation can be associated with cancer because of the increased power from decreasing the search space. Similarly, mutations that are clustered in a specific protein domain can be identified. Finally, if a variant has been found in one tumor type—for example, the canonical *KRAS* mutations found in

50% of melanomas—then when they occur in other tumor types, it is parsimonious to assume that they are oncogenic there as well even if they are rare.

At least a dozen methods have now been developed to identify genes (or sets of genes) that are selected by altering copy number changes. The principles for the detection of these genes are simple even if the implementations differ. First, copy number data are segmented to identify the locations of copy number change points using an algorithm such as CBS.[50] Once segmented, the data are normalized and germline copy number differences compared to the reference are removed. Finally, the data are analyzed to locate the genetic elements that are present in copy number aberrations more likely than expected by chance (e.g., STAC[51]). Copy number aberrations are thought to follow two distinct distributions: broad events that cover whole (or nearly whole) chromosome arms, and narrow events targeting much smaller regions (often fewer than 10 genes).[52] These software tools provide a list of the genes and chromosome arms that are frequently included in both broad and narrow events across many tumors. Although specific types of tumors have specific biases for (or against) specific genes/chromosome arms, many copy number aberrations are present in a diverse set of tumor types.[20] Methods to identify structural changes in the genome increasingly are based on the application of genome sequencing to both ends of genomic clones or fragments. The ends of each clone are then mapped onto a representation of the normal genome sequence. Structural aberrations are inferred when the paired ends of a clone map too close (signaling a deletion) or too far (signaling an insertion or translocation) along the genome. This approach was initially proposed for analysis of cloned sequences[53] but has become routine with the advent of massively parallel sequencing.[44] Once individual events are identified, standard statistical principles are then used to estimate the likelihood of seeing similar aberrations more frequently than expected by chance.

Organization into Pathways

A major challenge in cancer genomics is to understand how the ensemble of driver aberrations in an individual tumor influences its clinical and biological behavior. The remarkable genomic heterogeneity that exists in individual tumors can be managed to some extent by mapping aberrations onto pathways that influence the development of cancer hallmarks. The goal of these approaches is to reduce a dauntingly large number of functional genomic aberrations by mapping these onto a manageably small number of important pathways. Several approaches have been developed to organize omic information in ways that enable identification of pathways. We discuss gene-set enrichment approaches, pathway enrichment methods, and newer approaches that extend the repertoire of tools for pathway identification.

One of the most popular approaches is to use statistical tests on gene sets to implicate pathways that are deregulated by changes in the expression of that and related genes. A score is used to measure the degree to which each gene aberration is associated with the disease process, and then an enrichment analysis is performed using a large database of gene sets. For example, genes can be scored based on their length-normalized mutation frequency in a cohort, or assessed with more sophisticated analyses such as MutSig[54] or OncoDriveFM[55] to gauge how likely mutations in the gene provide a selective advantage to tumor cells. Once an appropriate score is applied to rank the genes, statistical tests can be used to identify enriched pathways. One approach is to threshold the list of genes to obtain those that are ranked toward the top of the list. These top-ranked genes then can be overlapped with each candidate pathway and a Fisher's exact or Hypergeometric test used to assess the statistical significance of the overlap to determine if it is higher than chance expectation. Overlap methods are implemented in web servers such as the DAVID[56] resource.

Gene Set Enrichment Analysis[57] (GSEA) compares the entire distribution of scores against a random background using a Kolmogorov-Smirnov–inspired test. Implicated pathways contain significantly more gene members with extreme (either high or low) scores. Gene set–based approaches are used frequently to test for enriched sets of genes, revealing important biological themes. However, the approach makes no use of known interactions between the tested genes. Thus, it is possible for a small but still significant subnetwork of genes to have significantly high scores and go undetected by these set-based approaches. In addition, all genes in a set are treated uniformly. However, some genes in the network may control many other genes while others are specialized effectors performing a specific cellular task in a limited set of conditions. Such genes may be weighted differently in the enrichment analysis to improve the sensitivity of the approach. Methods that incorporate notions of the local network organization of the scored genes can incorporate such intuitions and are discussed next.

"Master Regulator" algorithms attempt to identify genes residing at the logical "top" of predictive pathways whose manipulation would be expected to change the expression of downstream genes.[58] Signaling Pathway Impact Analysis (SPIA),[59] MARINa,[60] and GeneRank[61] are examples of algorithms in this class. The principle behind these algorithms can be likened to identifying authoritative pages on the Internet. A web page is considered authoritative if many other authoritative pages reference the page. The definition is necessarily recursive, forcing the algorithms to propagate information through the network to determine a solution. For master regulators, the links

in the network are reversed so that the methods home in on genes that control many other control genes, again in an iterative fashion. The approach has been used to propose master regulators for B-cell lymphoma.[60]

Another strategy is to search through large background networks for smaller subnetworks with a concentrated number of altered genes. Such subnetworks could represent pathways where disruptions in any of several gene members could interfere with the functioning of the pathway. These approaches make use of networks derived from high-throughput studies such as the collections of protein-protein interactions in BioGRID,[63] HPRD,[64] iREF,[65] and STRING[66] to identify novel pathways involved in tumorigenesis. These high-throughput sources can be used either alone or together with curated and directed signaling pathways found in resources like Reactome[67] and NCI's Protein Interaction Database.[68] Integrating somatic alterations and protein-protein interactions has the potential to provide a powerful means for cutting down false-positive rates present in either dataset because the sources of error are independent. Whether the subnetworks produced from these analyses are physiologically relevant is largely an open question but an area of intense activity.

HotNet[69] is a method for identifying enriched subnetworks, given a set of frequently altered mutations in a cohort. HotNet uses a heat-diffusion approach in which a mutated gene is considered to be a heat "source." The heat is allowed to dissipate on the background network for a short time interval so that genes neighboring the sources also heat up. Those residing close to multiple sources receive more heat than genes far away as an exponentially decaying function of the distance in the network. The algorithm then uses a hierarchical statistical test to identify significantly hot subnetworks. HotNet has been used to identify Notch-related pathways implicated in ovarian cystadenocarcinoma[3] and chromatin-remodeling pathways in clear-cell kidney carcinoma.[69a] These methods are especially well suited to the identification of subtype-specific subnetworks both within and across tumor types.

The Mutually Exclusive Modules (MEMo) algorithm[70] identifies novel networks from perturbation patterns observed across samples. This approach is based on the concept of mutual exclusivity—that is, mutation of a second gene in a cancer-related pathway provides no advantage in fitness beyond that provided by the first. The MEMo algorithm takes advantage of this mutual exclusivity property and builds an exhaustive graph of all approximate mutually exclusive gene pairs. Although the statistical significance of any two genes exhibiting such a mutually exclusive pattern is tenuous even in cohorts of hundreds of samples, the observation of a set of genes that all transitively share this property can be significant if the gene set is large enough (e.g., greater than three). MEMo leverages the significance of groups by exhaustively searching its network for subnetworks representing approximate cliques of sufficient size. Identified subnetworks are considered as candidate novel networks. New approaches in this vein, such as DENDRIX,[71] are also available that include additional statistical associations between genes beyond mutual exclusivity, such as the co-occurrence of mutational events.

The PARADIGM network analysis tool[72,73] uses information from multiple profiling measurements (copy number, mutations, transcription, etc.) to calculate inferred pathway activity levels (IPLs) for more than 1300 curated cell signaling pathways associated with specific recurrent aberrations, cancer types, or cancer subtypes. These data can be further combined into "superpathways" to identify subpathways therein whose activities differ between comparator populations (e.g., between transcriptional subtypes or between populations that differ in drug sensitivity). This approach has the advantage that it takes advantage of community knowledge of pathway architecture but has the disadvantage that the pathways may be inaccurate in some situations. PARADIGM has been used in several analyses,[3,4,6,73] demonstrating the power of inferred activities for identifying important tumor subtypes.

An extension of PARADIGM, PARADIGM-SHIFT[74] (PS), infers the impact of mutational events using network inference. Many mutations in advanced tumors are neutral passenger events resulting from the loss of genome integrity. In this background of a myriad spurious genomic perturbations, it is of interest to identify those that increase tumor fitness or that drive tumorigenesis forward. Several sequence-based methods are available to attack this important problem. However, an additional very important aspect, which has eluded computational analysis until very recently, is to predict whether the driving mutation causes a gain of function (GOF) or loss of function (LOF) to the protein. GOF mutations can lead to therapeutic manipulation because our biomedical tools often fare better at shutting down erroneously activated oncogenes than at introducing functional copies to rescue lost tumor suppressor activity. Pathway-based approaches offer promise in this area because the predicted activity of proteins in the pathway neighborhood can be inspected for signals of GOF and LOF. This is the approach taken by PS. PS predicts the impact of a mutation on the function of a protein by estimating the effects in the protein's pathway context. It uses two runs of the PARADIGM algorithm[72]—a "Targets-only" and "Regulators-only" run—to make this assessment. In the "Regulators-only" run, PS uses PARADIGM to infer the protein's activity after leaving connections only to the protein's upstream connections. In the "Targets-only" run, it estimates the activity of the protein with PARADIGM after leaving only the downstream connections intact. The difference, or "shift" between these two estimates provides an estimate of the loss or gain of function in the protein. PS has been successfully used to predict several known positive controls in

glioblastoma multiforme, lung squamous, and breast carcinomas.[74] One critical aspect for these network-based approaches is to select an informative local neighborhood around the protein, which can significantly influence overall accuracy. Thus machine-learning–based approaches such as the one described next could provide important synergies with these mutation-impact approaches.

Network-Induced Classification Kernels (NICK[75]) use networks to train support-vector machines to predict patient outcomes. Supervised machine learning is a well-established field that has contributed classification approaches for predicting discrete outcomes, and regression-based approaches for predicting continuous-valued outcomes. These methods face the "curse of dimensionality" problem when attempting to use the available large *feature spaces* (e.g., gene expression vectors) of high-throughput functional genomics to predict outcomes in a relatively small set (e.g., less than a thousand) of samples. Classifiers can suffer problems of robustness, reproducibility, and accuracy and can also misassess the importance of any single feature in the classification task. Only recently have approaches been developed to make use of a priori pathway knowledge for this task. NICK encodes the gene-gene interactions found in a network into the formulation of a support-vector machine classifier. The resulting method rewards selection of features that are adjacent in the network, thus resulting in solutions that are more robust, while maintaining classification accuracy. Methods such as NICK promise to stabilize solutions determined when the same task, such as predicting recurrence of disease, is applied to different datasets because the use of the same network should steer the solutions toward being comparable.

In summary, pathway- and network-based approaches represent a highly active area of current research in the analysis of cancer genomics datasets. New methods are still sorely needed to use the results of these approaches in a worthwhile effort to translate the findings to patient treatment. For example, the networks identified by these approaches could provide important insights into "Achilles' heel" attack points for cancer cells. We therefore need methods that can predict how a tumor might respond to a drug by simulating manipulations on such networks. An important antecedent to this, of course, is to prove that the networks capture enough of the salient features of a patient's tumor for it to be used as an "avatar" for in silico testing.

Experimental Approaches

The computational approaches just described attempt to predict functional genes based on their frequency, association with behavior, activation of pathways, and so forth. However, such approaches are limited by the number of samples available for computational assessment, the high heterogeneity within and between human tumors, and our imperfect understanding of the regulatory mechanisms that govern normal and malignant cell behavior. Thus, they serve to generate hypotheses that guide experimental validation in laboratory models.

Tumor Intrinsic Assessments

A wide range of in vitro and in vivo experimental systems are now available for functional assessment of the effects of genomic aberrations that occur in tumors and their impacts on therapeutic response. Given the extremely large number of aberrant genes and networks now being discovered, this summary focuses on methods that are sufficiently high throughput to allow "first pass" assessment of function. In general, these strategies assess the impact of manipulating cancer genes or networks on aspects of growth or immortalization and less frequently other aspects of cancer biology such as differentiation, angiogenesis, senescence, motility, and DNA repair activity. Biological systems now in widespread use for this purpose include well-characterized collections of immortalized cancer cell lines grown in two- or three-dimensional cultures,[73,76,77] cell lines such as IL-3–dependent, Ba/F3 hematopoietic cells that proliferate and survive in the absence of IL-3 when transfected with a constitutively active oncogene,[78,79] tumor xenograft collections,[80,81] genetically engineered murine models of cancer,[82,83] and mice subjected to transposon-mediated gene alteration leading to tumor formation.[84]

One powerful strategy for the manipulation of gene function introduces inhibitory RNA (RNAi) oligonucleotides into model organisms[85-87] to downregulate candidate genes or activated cancer regulatory networks. These RNAi precursors include short hairpin RNA (shRNA) oligonucleotides that are delivered through viral or bacterial vectors[87,88] and double-stranded RNA molecules, 20 to 25 base pairs in length, called *small interfering RNAs* (siRNAs)[85,89] that are transfected directly into target cells. Two general strategies are now commonly used to test the impact of RNAis in model organisms. One is to introduce libraries of RNAis that have been individually "barcoded" with unique nucleic acid sequences that can be identified by hybridization to oligonucleotide microarrays[89,90] or by massively parallel DNA sequencing.[91] The loss (selected against) or gain (selected for) of specific RNAis during growth is taken as evidence of the importance of the selected RNAis during growth. This approach has the advantage of enabling genome-wide screens at low cost but has the disadvantage of assessing only aspects of gene manipulation that affect aspects of cell growth. Another approach is to test the impact of siRNAs that

target individual genes in cells grown in microwells[92] or on cell spot microarrays.[93] The biological responses can be assessed by measuring changes in cancer-related properties relative to a control using assays that estimate cell number, or by using high-content imaging of cancer phenotypes such as DNA repair activity, differentiation, senescence, and motility after immunofluorescent staining for molecular surrogates for these phenotypes[94-96] and dynamic responses measured using time-lapse imaging.[97] These approaches have been useful in assessing the activity of specific pathways,[89] identifying genomic vulnerabilities that might be attacked therapeutically with single agents,[92] and developing strategies to combine therapeutic agents.[98,99]

Manipulation of gene function by transfection of cDNA libraries into nonmalignant cells also has been used to identify genes that enable the development of malignant phenotypes such as immortalization or colony-forming potential.[100] Another approach to cancer gene identification takes advantage of the tumorigenic integration of transposons into specific genes in murine model systems. The genomic locations in which transposons integrate are mapped by DNA sequencing approaches. Recurrent sites of integration identify genes that may contribute to tumor formation when activated or inactivated.[84,101]

Information about gene network function also can be inferred from measurements of responses of well-characterized cancer models to treatment with therapeutic agents that target specific genes or networks. Treatment with compounds in large collections of well-characterized cancer cell lines, for example, enables links to be established between specific aberrant genes or networks and biological responses using machine learning or pathway-based correlative strategies. The NCI's Discovery Therapeutic Program pioneered the use of cell lines to link omic features to response by measuring molecular features and responses to more than 100,000 compounds in a collection of about 60 cancer cell lines.[102] However, the NCI60 panel is of limited power in detecting subtype-specific responses because of the relatively sparse representation of specific cancer subtypes in the collection. This has led to the development of large collections of cell lines that represent the diversity within individual tumor types.[73,76] The Cancer Cell Line Encyclopedia (CCLE) and Sanger Cancer Cell Line (SCCL) projects have taken this approach to a higher level by assessing associations between responses to compounds in collections of approximately 800 cancer cell lines.[77,103] Several studies support the utility of in vitro testing in cell line panels. For example, in vitro model systems accurately show that (1) lung cancers with EGFR mutations respond to gefitinib,[104] (2) breast cancers with HER2/ERBB2 amplification respond to trastuzumab and/or lapatinib,[76,105] and (3) tumors with mutated or amplified BCR-ABL respond to imatinib mesylate.[106] Panels of xenografts also are now being developed for this purpose.[107]

Interaction with the Microenvironment

Much of cancer genomics research focuses on the tumor-intrinsic effects generated by aberrations in the tumors as discussed earlier. However, it is now apparent that the cancer-inducing functions of these aberrations are modified by signals from the microenvironments in which the cancer cells reside. Early research by Bissell and colleagues demonstrated that some extracellular microenvironments can counter the cancer-associated phenotypes generated by genomic aberrations[108]; Folkman and colleagues demonstrated the key role that angiogenesis plays in cancer progression.[109] Since then an explosion of research has illuminated many ways in which the microenvironment can affect aspects of cancer progression. These studies of the tumor-microenvironment interaction have been reviewed recently by Coussens and Hanahan.[110] They suggest that three general classes of cells from the microenvironment modulate cancer behavior in important ways: angiogenic vascular cells (AVCs), infiltrating immune cells (IICs), and cancer-associated fibroblastic cells (CAFs) as illustrated in Figure 24-2. They further suggest that the effects of these microenvironments influence aspects of cancer cell behavior including proliferation, growth, cell death, replicative immortality, inducing angiogenesis, energy metabolism, invasion, and metastasis. It is also apparent that the microenvironment influences responses to therapeutic agents—for example, by rendering cancer cells dormant so that they do not respond to cell-cycle active agents or by activating signaling therapy pathways. A challenge for the future will be to determine how diverse microenvironments experienced by metastatic cells influence the biological behavior of these cells—especially their responses to therapeutic interventions. Several model systems are now being developed to facilitate the study of the microenvironment on cancers. These include three-dimensional matrigel cultures,[111,112] two-dimensional systems engineered to carry many different proteins and growth factors from diverse microenvironments,[113,114] xenografts engineered to mirror important aspects of the human stroma,[115] and genetically engineered mice that model specific tumor intrinsic and extrinsic properties.[116]

Clinical Applications

Diagnosis and Detection

The manner in which normal tissue changes to malignant at the omic level is now being documented for a variety of

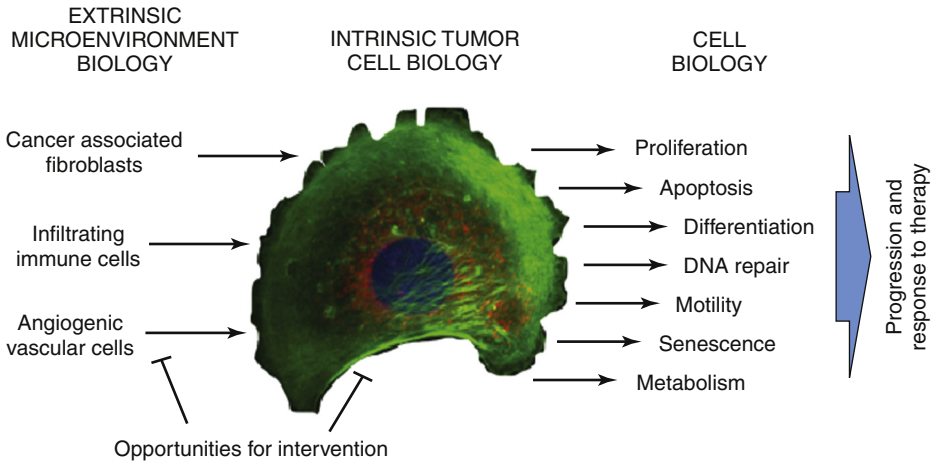


Figure 24-2 Interactions between tumor intrinsic and extrinsic features that influence cancer cell behavior and clinical outcome. *Cell image provided by Juha Rantala.*

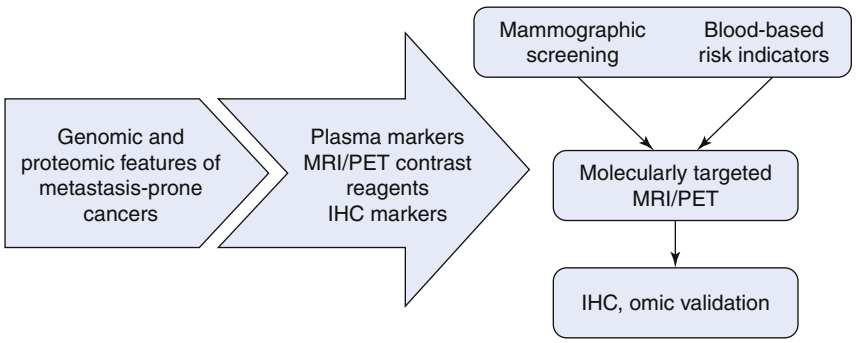


Figure 24-3 Schematic illustration of a genome-based approach to early cancer detection. *IHC*, Immunohistochemistry; *MRI*, magnetic resonance imaging; *PET*, positron emission tomography.

cancers by international efforts. These efforts will provide the basis for improved precision in cancer diagnosis and will show that most tumor types can be divided into subtypes that vary in outcome and often in response to therapy. For example, breast cancer tumors have been treated according to estrogen receptor status and according to whether HER2 is amplified for more than a decade. The advent of transcriptional profiling enabled breast cancers to be divided into six major transcriptional groups,[117,118] and adding information about genome copy number allows the definition of 10 subtypes.[119] Adding information about recurrent mutations or functional mutations will further subdivide these groups. Some of the associations with outcome are so strong that changes in cancer management practices have resulted. For example, several commercial assays that measure expression levels of multiple genes are now marketed that predict therapeutic benefit in breast cancer patients.[120-122] Since then, potentially useful diagnostic signatures have been developed for many cancer types including leukemia[123,124] and colorectal,[125] pancreatic,[126] and lung cancer.[127] More recently, expression levels of noncoding RNAs have been proven prognostic in cancers of the colon,[128] lung,[129] and bladder.[130] In some cases, these signatures are cancer type specific and as a result can be used to classify cancers of unknown origin.[131,132] Although most of these diagnostic signatures focus on molecular events that arise in the cancer, some reflect molecular features of the environments in which the tumors reside—for example, molecular signatures that originate in invading immune cells that influence tumor outcome.[133,134]

The identification of molecular features that are unique to cancers and associated with poor outcome also provides the basis for the development of assays that may identify cancers at high risk of progressing to metastatic disease at a time before they have metastasized so that they can still be treated successfully. Development of such assays would improve outcomes in patients afflicted with cancers of high metastatic potential and would reduce overtreatment of patients with low propensity for recurrence. These assays likely will be composed of a tiered combination of blood-based, anatomic, or histopathological assays with increasing sensitivity, specificity, and cost as illustrated in Figure 24-3.

Blood-based assays to date have focused on the detection of cancer-specific proteins and are low cost but also relatively low in sensitivity and specificity. Assays of prostate-specific antigen (PSA) for prostate cancer and CA-125 for ovarian cancer are prototypical, but omic analyses are now revealing a wide range of cancer-specific changes in gene expression and/or splicing that might increase the specificity of these tests. For example, powerful mass spectrometry techniques and computational analyses of genomic changes

are revealing increasing numbers of cancer-specific proteins that may be detected in blood.[135,136] In addition, it is now apparent that the ongoing process of tumor cell death leads to the appearance of tumor DNA fragments or microRNAs in peripheral blood or urine. Some of these tumor-derived DNA fragments carry aberrations such as mutations, translocations, and changes in methylation that are unique or very specific to the tumor. As a consequence, sensitive blood-based assays are now being developed to detect the presence of these sequences as an indication of the presence of cancer. Recent examples include an epigenetic marker panel for detecting lung cancer using cell-free serum DNA,[137] analysis of mutations in DNA isolated from plasma and stool of cancer patients,[138,139] detection of translocations as an indication of cancers of the prostate[140] or ovary,[141] and detection of genome copy-number changes as an indication of the presence of metastatic breast cancer.[139]

Anatomic cancer detection strategies based on the detection of specific molecular species using positron emission tomography (PET) and magnetic resonance imaging (MRI) are now being developed to enable the detection of cancer-specific genomic features. This requires the development of contrast reagents that make tumors and the aberrant microenvironments they produce visible when the tumors are still small and locally contained.[142] Genome profiling studies are revealing molecular features that are unique to early cancers. A variety of contrast reagents that target these are now being developed. These include reagents for the detection of estrogen receptor[143] and PSA[144]; a range of nanoparticles carrying affinity molecules that detect cancer-associated proteins[145-147]; and molecular features associated with cancer-associated stroma.[148]

Histological assessment of tissue samples taken from cancerous lesions has long been the gold standard for cancer detection and diagnosis. However, routine analyses of tissue sections stained with hematoxylin and eosin (H&E) currently do not provide sufficient information to distinguish between lesions of high and low malignant potential. Genome studies such as those described earlier are increasingly able to define molecular features associated with the most aggressive malignant lesions. This information is fueling the development of multiplex immunohistochemical assays and/or histologically targeted genomic assays that are better able to identify lesions at high risk of progressing.[149,150] These same assays also offer the potential of detecting isolated cancer cells that might be otherwise missed during an assessment of H&E-stained sections.

Therapeutic Targets and Predictive Markers

Discovery of strong driver aberrations that can be attacked with therapeutic benefit was an early motivating factor in the development of international genomics efforts.[151] Early discoveries showed that chronic myelogenous leukemias driven by the BCR-Abl tyrosine kinase could be effectively targeted by imatinib mesylate[152] and breast tumors driven by amplification of *HER2* could be effectively treated with trastuzumab.[153] Table 24-3[154] summarizes more recent driver genomic aberrations, the cancers in which they occur, and the successful therapeutic agents that attack them. This list will expand continuously as additional therapeutic agents for recurrent genomic aberrations are tested. Additional genes harboring genomic aberrations for which therapies are now being tested include *AKT1, PIK3CA, PTEN, MYC, VHL,* and *HRAS*.[151]

These studies are stimulating the development of a wealth of new therapeutic agents. Almost 900 small-molecule inhibitors and biological therapeutics are now under development for the treatment of human malignancies.[155] These agents target molecular features ranging from

Table 24-3 Genomic Aberrations, Therapeutic Agents, and Relevant Cancers[154]

Aberration	Therapeutic Compound	Cancer Type
BCR-Abl translocation	Imatinib mesylate	CML
ERBB2 amplification	Trastuzumab, lapatinib, pertuzumab	Breast cancer
KIT mutation	Imatinib mesylate	GIST
	Sunitinib	Dermatofibrosarcoma protuberans
PDGFRA mutation	Nilotinib	Hypereosinophilic syndrome
	Dasatinib	Melanoma
EGFR mutation	Gefitinib, erlotinib	Lung cancer
	Cetuximab	Bowel cancer
FLT2 mutation	PKC412, SU11248, CMT53518	AML, ALL
BRCA1/2 mutation	Olaparib, veliparib, iniparib	Breast/ovarian cancer
BRAF mutation	Vemurafenib	Melanoma, lung cancer
ALK translocation	Crizotinib	Lung cancer
ROS1 translocation	Crizotinib	Lung cancer
RET translocation	Vandetanib	Thyroid cancer
MET amplification	Crizotinib	Esophagogastric adenocarcinoma
KRAS	Cetuximab, panitumumab	Colorectal cancer
ER expression	Tamoxifen, aromatase inhibitors	Breast cancer

ALL, Acute lymphocytic leukemia; *AML*, acute myeloid leukemia; *CML*, chronic myelogenous leukemia; *GIST*, gastrointestinal stromal tumor.

broad-specificity conventional therapeutics to inhibitors that selectively target specific molecular aberrations and deregulated pathways. The general trend in drug development today is moving toward agents that are targeted toward pathways.[156]

The traditional path to the clinic for new cancer drugs is to test them in phased trials in the metastatic setting, followed by testing in randomized Phase III registration trials in the adjuvant setting. This approach requires a substantial investment in time, number of patients, and money. The U.S. Food and Drug Administration (FDA) has published draft guidance for using pathological complete response in neoadjuvant treatment for accelerated approval in high-risk breast cancer, which would dramatically accelerate the approval process.[157] Although a step forward, this approach has the weakness that drugs that are effective only in a small population of patients may be discarded because of lack of apparent efficacy. Biomarkers that predict response to therapy would enable identification of these small subpopulations so that they can be targeted early in the clinical trials. As described earlier, this can be accomplished by developing initial insights about subpopulation specificity using preclinical models of aspects of tumor-intrinsic and tumor-extrinsic heterogeneity that influence responses.

It is also becoming clear that specific regulatory pathways can differ among cancer subtypes so that these subtypes respond differently to targeted and nontargeted therapies. It has long been recognized, for example, that estrogen-receptor–positive (ER^+) breast cancers will respond well to selective estrogen response modifiers[158] and that a subset of prostate cancers is responsive to inhibitors of androgen receptors.[159] However, it now appears that most anticancer agents will be preferentially active in cancer subtypes defined according to their genomic characteristics.[73] The explanation for this seems to be that the use of molecular pathways that regulate cell behavior (and response to therapy) differs among subtypes. Efforts in the TCGA project and other international genomics efforts are defining subtypes in most anatomically defined cancers that can be considered for stratification of therapeutic response. Full use of this information will require the development of approved molecular assays that can stratify patients according to subtype.

Summary

International efforts are now defining the genomic and epigenomic landscapes of most major tumor types. The first set of cross-tumor (a.k.a. "Pan-Cancer") studies are now emerging to help delineate core and lineage-specific contributors of the disease.[160] These studies are revealing a few strong driver aberrations in each cancer type and many—sometimes thousands—of aberrations of unknown consequence. Much work remains to determine which of these contribute to the pathophysiology of each cancer type, but it is already clear that these analyses will have a profound effect on the way most cancers are managed. Aspects of cancer management that will benefit include early detection of the most lethal cancers, identification of recurrently aberrant genes and networks for high-priority therapeutic attack, and development of molecular markers that predict response to gene- or network-targeted therapies.

Key References

2. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455:1061-1068.
3. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609-615.
4. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487:330-337.
5. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012;489:519-525.
6. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
7. Wood LD, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318:1108-1113.
8. Shah SP, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486:395-399.
9. Russnes HG, et al. Genomic architecture characterizes tumor progression paths and fate in breast cancer patients. *Sci Transl Med*. 2010;2:38ra47.
10. Stephens PJ, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature*. 2009;462:1005-1010.
11. Jones S, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806 .
12. Prensner JR, et al. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat Biotechnol*. 2011;29:742-749.
16. Chapman MA, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011;471:467-472.
17. Ding L, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506-510.
19. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
23. Hodis E, et al. A landscape of driver mutations in melanoma. *Cell*. 2012;150:251-263.
29. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome—components and functional correlates. *Genes Dev*. 2006;20:3215-3231.
33. Durinck S, et al. Temporal dissection of tumorigenesis in primary cancers. *Cancer Discov*. 2011;1:137-143.
34. Vogelstein B, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med*. 1988;319:525-532.
44. Chen K, et al. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nat Methods*. 2009;6:677-681 1363.
46. Trapnell C, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012;7:562-578.

50. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics*. 2004;5:557-572.
52. Beroukhim R, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
53. Volik S, et al. End-sequence profiling: sequence-based analysis of aberrant genomes. *Proc Natl Acad Sci U S A*. 2003;100:7696-7701.
56. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
57. Subramanian A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102:15545-15550.
60. Lefebvre C, et al. A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol*. 2010;6:377.
66. Franceschini A, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acid Res*. 2013;41:D808-D815.
67. Matthews L, et al. Reactome knowledgebase of human biological pathways and processes. *Nucleic Acid Res*. 2009;37:D619-D622.
69a. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature*. 2013; 499:43-49.
72. Vaske CJ, et al. Inference of patient-specific pathway activities from multi-dimensional cancer genomics data using PARADIGM. *Bioinformatics*. 2010; 26:i237-i245 btq182 [pii]10.1093/bioinformatics/btq182.
73. Heiser LM, et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:2724-2729.
77. Barretina J, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-607.
82. Chen Z, et al. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature*. 2012;483:613-617.
84. Copeland NG, Jenkins NA. Harnessing transposons for cancer gene discovery. *Nat Rev Cancer*. 2010;10:696-706.
98. Rehman FL, Lord CJ, Ashworth A. Synthetic lethal approaches to breast cancer therapy. *Nat Rev Clin Oncol*. 2010;7:718-724.
103. Garnett MJ, et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012;483:570-575.
104. Paez JG, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
110. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21:309-322.
116. Marks C. Mouse Models of Human Cancers Consortium (MMHCC) from the NCI. *Dis Model Mech*. 2009;2:111.
118. Perou CM, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.
134. DeNardo DG, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1:54-67.
139. Forshew T, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012;4 136ra168.
151. Dancey JE, Bedard PL, Onetto N, Hudson TJ. The genetic basis for cancer treatment decisions. *Cell*. 2012;148:409-420.
152. Druker BJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344: 1031-1037.
153. Pegram MD, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol*. 2098;16:2659-2671.
156. Sawyers C. Targeted cancer therapy. *Nature*. 2004;432:294-297.
160. PMID:24071849.

References

1. Bhat KP, et al. The transcriptional coactivator TAZ regulates mesenchymal differentiation in malignant glioma. *Genes Dev*. 2011;25:2594-2609.
2. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455:1061-1068.
3. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609-615.
4. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487:330-337.
5. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012;489:519-525.
6. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
7. Wood LD, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007;318:1108-1113.
8. Shah SP, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486:395-399.
9. Russnes HG, et al. Genomic architecture characterizes tumor progression paths and fate in breast cancer patients. *Sci Transl Med*. 2010;2:38ra47.
10. Stephens PJ, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature*. 2009;462:1005-1010.
11. Jones S, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806.
12. Prensner JR, et al. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat Biotechnol*. 2011;29:742-749.
13. Liu J, et al. Genome and transcriptome sequencing of lung cancers reveal diverse mutational and splicing events. *Genome Res*. 2012;22:2315-2327.
14. Dalgliesh GL, et al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. *Nature*. 2010;463:360-363.
15. Berger MF, et al. Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature*. 2012;485:502-506.
16. Chapman MA, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011;471:467-472.
17. Ding L, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature*. 2012;481:506-510.
18. Chin L, Gray JW. Translating insights from the cancer genome into clinical practice. *Nature*. 2008;452:553-563.
19. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
20. Kim TM, et al. Functional genomic analysis of chromosomal aberrations in a compendium of 8000 cancer genomes. *Genome Res*. 2013;23:217-227.
21. Curtis C, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486:346-352.
22. Pugh TJ, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45:279-284.
23. Hodis E, et al. A landscape of driver mutations in melanoma. *Cell*. 2012;150:251-263.
24. Chin K, et al. In situ analyses of genome instability in breast cancer. *Nat Genet*. 2004;36:984-988.
25. Artandi SE, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature*. 2000;406:641-645.
26. You JS, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell*. 2012;22:9-20.
27. Sharpless NE, DePinho RA. p53: good cop/bad cop. *Cell*. 2002;110:9-12.
28. Venkatesan RN, Bielas JH, Loeb LA. Generation of mutator mutants during carcinogenesis. *DNA Repair (Amst)*. 2006;5:294-302.
29. Ting AH, McGarvey KM, Baylin SB. The cancer epigenome—components and functional correlates. *Genes Dev*. 2006;20:3215-3231.
30. Davies H, et al. Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Res*. 2005;65:7591-7595.
31. Pleasance ED, et al. A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature*. 2010;463:184-190.
32. Yachida S, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature*. 2010;467:1114-1117.
33. Durinck S, et al. Temporal dissection of tumorigenesis in primary cancers. *Cancer Discov*. 2011;1:137-143.
34. Vogelstein B, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med*. 1988;319:525-532.
35. Attolini CS, et al. A mathematical framework to determine the temporal sequence of somatic genetic events in cancer. *Proc Natl Acad Sci U S A*. 2010;107:17604-17609.
36. Cheng YK, et al. A mathematical methodology for determining the temporal order of pathway alterations arising during gliomagenesis. *PLoS Comput Biol*. 8, e1002337; 2012.
37. Nik-Zainal S, et al. The life history of 21 breast cancers. *Cell*. 2012;149:994-1007.
38. Fullgrabe J, Kavanagh E, Joseph B. Histone onco-modifications. *Oncogene*. 2011;30:3391-3403.
39. van Engeland M, Derks S, Smits KM, Meijer GA, Herman JG. Colorectal cancer epigenetics: complex simplicity. *J Clin Oncol*. 2011;29:1382-1391.
40. Luijsterburg MS, van Attikum H. Chromatin and the DNA damage response: the cancer connection. *Mol Oncol*. 2011;5:349-367.
41. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754-1760.
42. Abecasis GR, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491:56-65.
43. Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. *Bioinformatics*. 2009;25:2865-2871.
44. Chen K, et al. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nat Methods*. 2009;6:677-681.
45. Rausch T, et al. DELLY: structural variant discovery by integrated paired-end and split-read analysis. *Bioinformatics*. 2012;28:i333-i339.
46. Trapnell C, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc*. 2012;7:562-578.
47. Weng L, et al. MicroRNA profiling of clear cell renal cell carcinoma by whole-genome small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens. *J Pathol*. 2010;222:41-51.
48. Lee C, Kikyo N. Strategies to identify long noncoding RNAs involved in gene regulation. *Cell Biosci*. 2012;2:37.
49. Liu L, De S, Michor F. DNA replication timing and higher-order nuclear organization determine single-nucleotide substitution patterns in cancer genomes. *Nat Commun*. 2013;4:1502.
50. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics*. 2004;5:557-572.
51. Diskin SJ, et al. STAC: A method for testing the significance of DNA copy number aberrations across multiple array-CGH experiments. *Genome Res*. 2006;16:1149-1158.
52. Beroukhim R, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010;463:899-905.
53. Volik S, et al. End-sequence profiling: sequence-based analysis of aberrant genomes. *Proc Natl Acad Sci U S A*. 2003;100:7696-7701.
54. Cibulskis K, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. 2013;31:213-219.
55. Gonzalez-Perez A, Lopez-Bigas N. Functional impact bias reveals cancer drivers. *Nucleic Acid Res*. 2012;40:e169.
56. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 2009;4:44-57.
57. Subramanian A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102:15545-15550.
58. Page L, Brin S, Motwani R, Winograd T. The PageRank citation ranking: bringing order to the web. http://infolab.stanford.edu/~backrub/pageranksub.ps. 1999.
59. Tarca AL, et al. A novel signaling pathway impact analysis. *Bioinformatics*. 2009;25:75-82.

60. Lefebvre C, et al. A human B-cell interactome identifies MYB and FOXM1 as master regulators of proliferation in germinal centers. *Mol Syst Biol*. 2010;6:377.
61. Morrison JL, Breitling R, Higham DJ, Gilbert DR. GeneRank: using search engine technology for the analysis of microarray experiments. *BMC Bioinformatics*. 2005;6:233.
62. REFERENCE DELETED IN PROOFS.
63. Chatr-Aryamontri R, et al. The BioGRID interaction database: 2013 update. *Nucleic Acid Res*. 2013;41:D816-D823.
64. Mathivanan S, et al. Human Proteinpedia enables sharing of human protein data. *Nat Biotechnol*. 2008;26:164-167.
65. Turner B, et al. iRefWeb: interactive analysis of consolidated protein interaction data and their supporting evidence. *Database (Oxford)*. 2010, baq023.
66. Franceschini A, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acid Res*. 2013;41: D808-D815.
67. Matthews L, et al. Reactome knowledgebase of human biological pathways and processes. *Nucleic Acid Res*. 2009;37:D619-D622.
68. Schaefer CF, et al. PID: the Pathway Interaction Database. *Nucleic Acid Res*. 2009;37:D674-D679.
69. Vandin F, Upfal E, Raphael BJ. Algorithms for detecting significantly mutated pathways in cancer. *J Comput Biol*. 2011;18:507-522.
69a. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013, 499:43-9.
70. Ciriello G, Cerami E, Sander C, Schultz N. Mutual exclusivity analysis identifies oncogenic network modules. *Genome Res*. 2012;22:398-406.
71. Vandin F, Upfal E, Raphael BJ. De novo discovery of mutated driver pathways in cancer. *Genome Res*. 2012;22:375-385.
72. Vaske CJ, et al. Inference of patient-specific pathway activities from multi-dimensional cancer genomics data using PARADIGM. *Bioinformatics*. 2010;26:i237-i245 btq182 [pii]10.1093/bioinformatics/btq182.
73. Heiser LM, et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc Natl Acad Sci U S A*. 2012;109:2724-2729.
74. Ng S, et al. PARADIGM-SHIFT predicts the function of mutations in multiple cancers using pathway impact analysis. *Bioinformatics*. 2012;28: i640-i646.
75. Lavi O, Dror G, Shamir R. Network-induced classification kernels for gene expression profile analysis. *J Comput Biol*. 2012;19:694-709.
76. Neve RM, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. 2006;10:515-527 S1535-6108[06]0314-X [pii]10.1016/j.ccr. 2006.10.008.
77. Barretina J, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-607.
78. Loriaux MM, et al. High-throughput sequence analysis of the tyrosine kinome in acute myeloid leukemia. *Blood*. 2008;111:4788-4796.
79. Warmuth M, Kim S, Gu XJ, Xia G, Adrian F. Ba/F3 cells and their use in kinase drug discovery. *Curr Opin Oncol*. 2007;19:55-60.
80. Kerbel RS. Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived-but they can be improved. *Cancer Biol Ther*. 2003;2:S134-S139.
81. Sausville EA, Burger AM. Contributions of human tumor xenografts to anticancer drug development. *Cancer Res*. 2006;66:3351-3354; discussion 3354.
82. Chen Z, et al. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature*. 2012;483:613-617.
83. Nardella C, Lunardi A, Patnaik A, Cantley LC, Pandolfi PP. The APL paradigm and the "co-clinical trial" project. *Cancer Discov*. 2011;1:108-116.
84. Copeland NG, Jenkins NA. Harnessing transposons for cancer gene discovery. *Nat Rev Cancer*. 2010;10:696-706.
85. Chang K, Marran K, Valentine A, Hannon GJ. RNAi in cultured mammalian cells using synthetic siRNAs. *Cold Spring Harb Protoc*. 2012:957-961.
86. Dow LE, et al. A pipeline for the generation of shRNA transgenic mice. *Nat Protoc*. 2012;7:374-393.
87. Muerdter F, et al. Production of artificial piRNAs in flies and mice. *RNA*. 2012;18:42-52.
88. Silva JM, et al. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet*. 2005;37:1281-1288.
89. Berns K, et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature*. 2004;428:431-437.
90. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science*. 2002;296:550-553.
91. Sims D, et al. High-throughput RNA interference screening using pooled shRNA libraries and next generation sequencing. *Genome Biol*. 2011;12:R104.
92. Cheung HW, et al. Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci U S A*. 2011;108:12372-12377.
93. Rantala JK, et al. A cell spot microarray method for production of high density siRNA transfection microarrays. *BMC Genomics*. 2011;12:162.
94. Bjorkman M, et al. Systematic knockdown of epigenetic enzymes identifies a novel histone demethylase PHF8 overexpressed in prostate cancer with an impact on cell proliferation, migration and invasion. *Oncogene*. 2012;31:3444-3456.
95. Krausz E, Korn K. High-content siRNA screening for target identification and validation. *Expert Opin Drug Discov*. 2008;3:551-564.
96. Krausz E. High-content siRNA screening. *Mol Biosyst*. 2007;3:232-240.
97. Neumann B, et al. High-throughput RNAi screening by time-lapse imaging of live human cells. *Nat Methods*. 2006;3:385-390.
98. Rehman FL, Lord CJ, Ashworth A. Synthetic lethal approaches to breast cancer therapy. *Nat Rev Clin Oncol*. 2010;7:718-724.
99. Turner NC, et al. A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor. *EMBO J*. 2008;27:1368-1377.
100. Wan D, et al. Large-scale cDNA transfection screening for genes related to cancer development and progression. *Proc Natl Acad Sci U S A*. 2004;101: 15724-15729.
101. Copeland NG, Jenkins NA. Deciphering the genetic landscape of cancer—from genes to pathways. *Trends Genet*. 2009;25:455-462.
102. Bussey KJ, et al. Integrating data on DNA copy number with gene expression levels and drug sensitivities in the NCI-60 cell line panel. *Mol Cancer Ther*. 2006;5:853-867.
103. Garnett MJ, et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012;483:570-575.
104. Paez JG, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
105. Konecny GE, et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res*. 2006;66:1630-1639.
106. Scappini B, et al. Changes associated with the development of resistance to imatinib (STI571) in two leukemia cell lines expressing p210 Bcr/Abl protein. *Cancer*. 2004;100:1459-1471.
107. Julien S, et al. Characterization of a large panel of patient-derived tumor xenografts representing the clinical heterogeneity of human colorectal cancer. *Clin Cancer Res*. 2012;18:5314-5328.
108. Rizki A, et al. A human breast cell model of preinvasive to invasive transition. *Cancer Res*. 2008;68:1378-1387.
109. Kieran MW, Kalluri R, Cho YJ. The VEGF pathway in cancer and disease: responses, resistance, and the path forward. *Cold Spring Harb Perspect Med*. 2012;2:a006593. doi: 10.1101/cshperspect.a006593.
110. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;21:309-322.
111. Nelson CM, Bissell MJ. Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation. *Semin Cancer Biol*. 2005;15:342-352.
112. Debnath J, Brugge JS. Modelling glandular epithelial cancers in three-dimensional cultures. *Nat Rev Cancer*. 2005;5:675-688.
113. Lin CH, Lee JK, LaBarge MA. Fabrication and use of microenvironment microarrays (MEArrays). *J Vis Exp*. 2012(68).
114. Wilson TR, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature*. 2012;487:505-509.
115. Kuperwasser C, et al. Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci U S A*. 2004;101:4966-4971.
116. Marks C. Mouse Models of Human Cancers Consortium (MMHCC) from the NCI. *Dis Model Mech*. 2009;2:111.
117. Prat A, Parker JS, Fan C, Perou CM. PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. *Breast Cancer Res Treat*. 2012;135:301-306.
118. Perou CM, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.

119. Stephens PJ, et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature*. 2012;486:400-404.
120. NSABP study confirms oncotype DX predicts chemotherapy benefit in breast cancer patients. *Oncology (Williston Park)*. 2006;20:789-790.
121. Ross JS, Hatzis C, Symmans WF, Pusztai L, Hortobagyi GN. Commercialized multigene predictors of clinical outcome for breast cancer. *Oncologist*. 2008;13:477-493.
122. Glas AM, et al. Converting a breast cancer microarray signature into a high-throughput diagnostic test. *BMC Genomics*. 2006;7:278.
123. Dave SS, et al. Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med*. 2006;354:2431-2442.
124. Litvinov IV, Jones DA, Sasseville D, Kupper TS. Transcriptional profiles predict disease outcome in patients with cutaneous T-cell lymphoma. *Clin Cancer Res*. 2010;16:2106-2114.
125. Nannini M, et al. Gene expression profiling in colorectal cancer using microarray technologies: results and perspectives. *Cancer Treat Rev*. 2009;35:201-209.
126. Collisson EA, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med*. 2011;17:500-503 nm.2344 [pii]10.1038/nm.2344.
127. Roepman P, et al. An immune response enriched 72-gene prognostic profile for early-stage non-small-cell lung cancer. *Clin Cancer Res*. 2009;15:284-290.
128. Chen HY, et al. miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. *Cancer Res*. 2012;72:3631-3641.
129. Boeri M, Pastorino U, Sozzi G. Role of microRNAs in lung cancer: microRNA signatures in cancer prognosis. *Cancer J*. 2012;18:268-274.
130. Rosenberg E, et al. Predicting progression of bladder urothelial carcinoma using microRNA expression. *BJU Int*. 2013;112:1027-1034.
131. Varadhachary GR, et al. Prospective gene signature study using microRNA to identify the tissue of origin in patients with carcinoma of unknown primary. *Clin Cancer Res*. 2011;17:4063-4070.
132. Bender RA, Erlander MG. Molecular classification of unknown primary cancer. *Semin Oncol*. 2009;36:38-43.
133. Hsu DS, et al. Immune signatures predict prognosis in localized cancer. *Cancer Invest*. 2010;28:765-773.
134. DeNardo DG, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1:54-67.
135. Anderson NL, et al. A human proteome detection and quantitation project. *Mol Cell Proteomics*. 2009;8:883-886.
136. Drake PM, et al. Lectin chromatography/mass spectrometry discovery workflow identifies putative biomarkers of aggressive breast cancers. *J Proteome Res*. 2012;11:2508-2520.
137. Carvalho AL, et al. Detection of promoter hypermethylation in salivary rinses as a biomarker for head and neck squamous cell carcinoma surveillance. *Clin Cancer Res*. 2011;17:4782-4789.
138. Diehl F, et al. Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. *Gastroenterology*. 2008;135:489-498.
139. Forshew T, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci Transl Med*. 2012;4 136ra168.
140. Morris DS, Tomlins SA, Montie JE, Chinnaiyan AM. The discovery and application of gene fusions in prostate cancer. *BJU Int*. 2008;102:276-282.
141. Salzman J, et al. ESRRA-C11orf20 is a recurrent gene fusion in serous ovarian carcinoma. *PLoS Biol*. 2011;9 e1001156.
142. Nahrendorf M, et al. Hybrid PET-optical imaging using targeted probes. *Proc Natl Acad Sci U S A*. 2010;107:7910-7915.
143. Lee JH, et al. Synthesis and biological evaluation of two agents for imaging estrogen receptor beta by positron emission tomography: challenges in PET imaging of a low abundance target. *Nucl Med Biol*. 2012;39:1105-1116.
144. Evans MJ, et al. Noninvasive measurement of androgen receptor signaling with a positron-emitting radiopharmaceutical that targets prostate-specific membrane antigen. *Proc Natl Acad Sci U S A*. 2011;108:9578-9582.
145. Condeelis J, Weissleder R. In vivo imaging in cancer. *Cold Spring Harb Perspect Biol*. 2010;2 a003848.
146. Holland JP, et al. Annotating MYC status with 89Zr-transferrin imaging. *Nat Med*. 2012;18:1586-1591.
147. Jagoda EM, et al. Immuno-PET of the hepatocyte growth factor receptor Met using the 1-armed antibody onartuzumab. *J Nucl Med*. 2012;53:1592-1600.
148. Nabavizadeh N, et al. Topographic enhancement mapping of the cancer-associated breast stroma using breast MRI. *Integr Biol (Camb)*. 2011;3:490-496.
149. Cuzick J, et al. Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. *J Clin Oncol*. 2011;29:4273-4278.
150. Bordeaux JM, et al. Quantitative in situ measurement of estrogen receptor mRNA predicts response to tamoxifen. *PLoS One*. 2012;7 e36559.
151. Dancey JE, Bedard PL, Onetto N, Hudson TJ. The genetic basis for cancer treatment decisions. *Cell*. 2012;148:409-420.
152. Druker BJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-1037.
153. Pegram MD, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol*. 2098;16:2659-2671.
154. Mills GB. An emerging toolkit for targeted cancer therapies. *Genome Res*. 2012;22:177-182.
155. Castellan J. http://www.phrma.org/sites/default/files/1000/medicinesindevelopmentcancer2011_0.pdf. 2011.
156. Sawyers C. Targeted cancer therapy. *Nature*. 2004;432:294-297.
157. Prowell TM, Pazdur R. Pathological complete response and accelerated drug approval in early breast cancer. *N Engl J Med*. 2012;366:2438-2441.
158. Peng J, Sengupta S, Jordan VC. Potential of selective estrogen receptor modulators as treatments and preventives of breast cancer. *Anticancer Agents Med Chem*. 2009;9:481-499.
159. Friedlander TW, Ryan CJ. Targeting the androgen receptor. *Urol Clin North Am*. 2012;39:453-464.
160. PMID:24071849.

Albert Gough, Timothy Lezon, James R. Faeder, Chakra Chennubhotla,
Robert F. Murphy, Rebecca Critchley-Thorne, and D. Lansing Taylor

25

High-Content Analysis with Cellular and Tissue Systems Biology: A Bridge between Cancer Cell Biology and Tissue-Based Diagnostics

High-Content Analysis (HCA)

Background on Fluorescence Imaging in Cancer Biology, Drug Discovery, and Diagnostics

Cancer displays significant genetic and nongenetic heterogeneity.[1-4] Tumors are integrated tissue systems of interacting malignant cells, stem cells, and stromal components, including immune cells, fibroblasts, endothelial cells, and nerve cells (Figure 25-1).[5-9] Immune cells and other stromal components play cooperative roles in tumor development and metastasis and influence responses to therapies. The stromal phenotype and functions are strongly associated with disease progression and clinical outcome in cancer. Leukocytes are attracted into tumors by chemokines and can both protect the tumor from antitumor immunity and promote tumor progression via stimulating angiogenesis and tumor cell migration.[10-12] Tumors can render infiltrating immune cells anergic/nonresponsive[13,14] or drive such cells into apoptosis.[15] Tumor cell heterogeneity, the complexity of the tumor system, and the vital interactions of tumor cells with multiple components of the stroma highlight the need for a "tissue systems biology" approach to cancer diagnostics, which combines multiplexed biomarker measurements in the context of the tissue architecture and tumor cell function (Table 25-1)* with informatics tools to classify individual patients according to disease subtype, recurrence, and responses to therapies.[16]

Fluorescence-based imaging technologies have been applied to models of cancer and patient samples for many years. The applications have spanned the range from in vitro studies in single cells,[17,18] populations of cells,[18-24] mixed cell populations,[25-27] three-dimensional (3D) tumor models,[28,29] and pathology of patient tumor samples,[16,30] as well as imaging cells within mouse tumor models,[31-33] including antitumor immune responses,[31,34] dynamics of cancer growth and invasion,[31,35] tumor angiogenesis and regression,[31,36] and tumor cell movements.[37,38] Furthermore, applications in drug discovery have been performed in cells[39,40] and in small experimental organisms[41] including yeast,[42-45] *Caenorhabditis elegans*,[46-48] *Drosophila*,[49-52] and zebrafish,[53-56] as well as monitoring tumors in rodent models[33,57-59] with whole-body imaging of small mammals.[57,60]

The present chapter focuses on the application of high-content analysis (HCA) to populations of cells, more complex tumor models, and in vitro and patient samples where large image datasets can be created and explored with computational and systems biology tools to create a bridge between cancer cell biology and tissue-based diagnostics. The investigation and integration of the continuum of single cells, cell populations, 3D tumor models, and patient samples is needed to define the molecular basis of cancer.

HCA, originally termed high-content screening (HCS), is a platform technology created in the 1990s to automatically image, analyze, store, and mine large image datasets based primarily on fluorescence imaging microscopy, although transmitted light is an option.[61,62] HCA harnesses advances in automation of microscopy, image processing, image analysis, fluorescence-based reagents, automation of sample preparation, and relational databases (Figure 25-2).[61-64] There have been numerous books[65-67] and reviews on the applications of HCA in basic biomedical research, drug discovery/development, and diagnostics.[39,68-72] A broad range of fluorescence-based reagents for both live cell, kinetic studies and fixed-endpoint investigations have also been reviewed in detail.[63] The major types of reagents, readouts, and selected on-line databases are listed in

*Table 25-1 includes citations for References 214, 224, and 294 through 331.

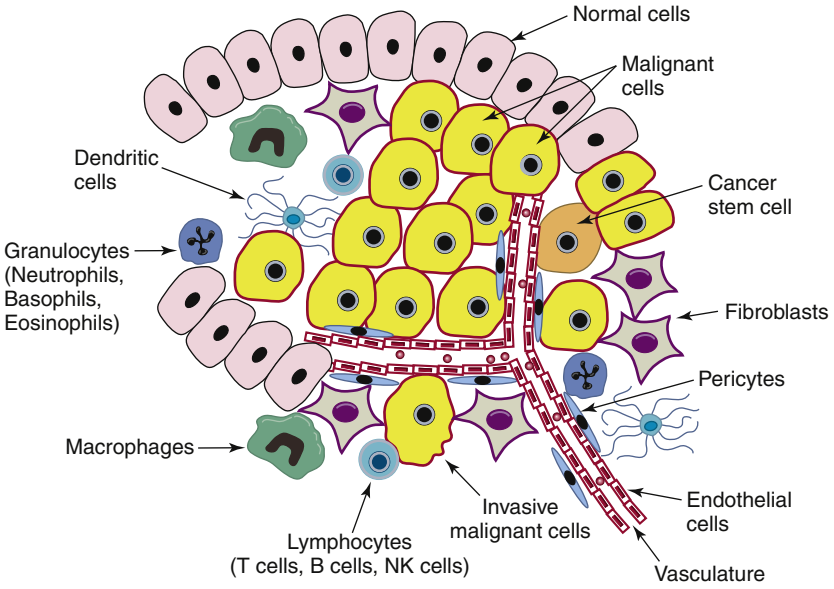


Figure 25-1 Solid tumors are systems within the human system The tumor system includes normal tissue/organ cells *(pink)*, tumor cells usually containing a range of genetic alterations *(yellow)*, and cancer stem cells *(orange)*, along with immune cells (e.g., dendritic cells, granulocytes, macrophages, lymphocytes) and other stromal cells, such as fibroblasts and vasculature, that all play a part in tumor development, progression, metastasis, and response to therapy, based in part on their spatial relationships.

Table 25-1 Key Tumor System Processes and Biomarkers

System Process	Example Biomarkers	References
Proliferation	Ki-67, Aurora A kinase	294, 295
Apoptosis	p53, Apo-1/Fas, FasL, TRAIL receptors, caspases, pAKT, Survivin, MCL-1, Bcl-2	296-303
Cell cycle control	p53, p21, p27, p16, cyclins D1, E	304-310
Adhesion	E-cadherin, beta-catenin, CD44, CD24, Claudin-1	311-315
Migration/motility	CXCR4, alpha6beta4 integrin, Net1, matrix metalloproteinases	316-318
Angiogenesis	VEGF, Flt-4, HIF-1alpha, pericyte markers	319-323
Immune responses	CD68, CD45RO, CD3zeta, CD4, CD8, PD-L1, FOXP3, CD1a, cytokines	224, 324-327
Inflammation	NF-kappaB, COX2, CSF-1R	214, 328, 329
Fibroblasts	Fibroblast activation protein-alpha, PDGF-beta	330, 331

Adapted from Critchley-Thorne RJ, Miller SM, Taylor DL, et al. Applications of cellular systems biology in breast cancer patient stratification and diagnostics. *Comb Chem High Throughput Screen*. 2009;12:860-869.

Table 25-2.* Extensive lists of additional reagent sources can be found online[73,74] and in published catalogues.[75]

Although the development of HCA has focused on the application of fluorescent probes, chromogenic probes continue to be used extensively for labeling tissue sections. Table 25-3** compares the advantages and disadvantages of fluorescent and chromogenic probes. Most HCA systems are optimized for the use of fluorescent probes, principally for their high sensitivity, high specificity, broad range of cellular functional readouts, broad range of wavelengths for multiplexing, and ability to engineer cells to express fluorescent proteins and biosensors. Because HCA makes use of automated imaging and quantitative image analysis, there is no need for direct viewing of the labeled specimen, and once the images are acquired, there is no further need for the specimen other than for institutional or clinical requirements. In traditional pathology, on the other hand, chromogenic probes have some advantages. The human brain is still the most sophisticated and reliable image processor for the interpretation of small numbers of images.[76] Readily available, low-cost chromogenic probes provide stable and dense labeling for visualization in a transmitted light microscope or by digital image pathology, while simultaneously viewing the contextual morphology of the cells. Although providing somewhat lower resolution and more limited multiplexing than fluorescent probes, chromogenic probes still provide a good labeling strategy where one to three biomarkers per slide can be useful.

Success in the Human Genome Project demanded tools to define the functions of the coding and noncoding portions of the genome, to define the dynamic interplay of cellular constituents within and between cells, and to characterize subpopulations, as well as to define the relationships between populations of cells in higher order biological systems. The field was named *cellomics*, and the platform technology was named *HCA*. HCA harnesses the ability to implement combinatorial treatments on large sample sizes by using microplates, patterned microarrays[77] and microfluidic devices[78] for cells, microplates for small organisms, and mounted sections/microarrays for tissues. These large sample sizes are required for statistical analyses and exploration by computational and systems biology.[16,21,63,79,80]

*Table 25-2 includes citations for References 61, 63, 68, 69, 74, 75, 110, 133, 197, and 332 through 340.
**Table 25-3 includes citations for References 177 and 341 through 344.

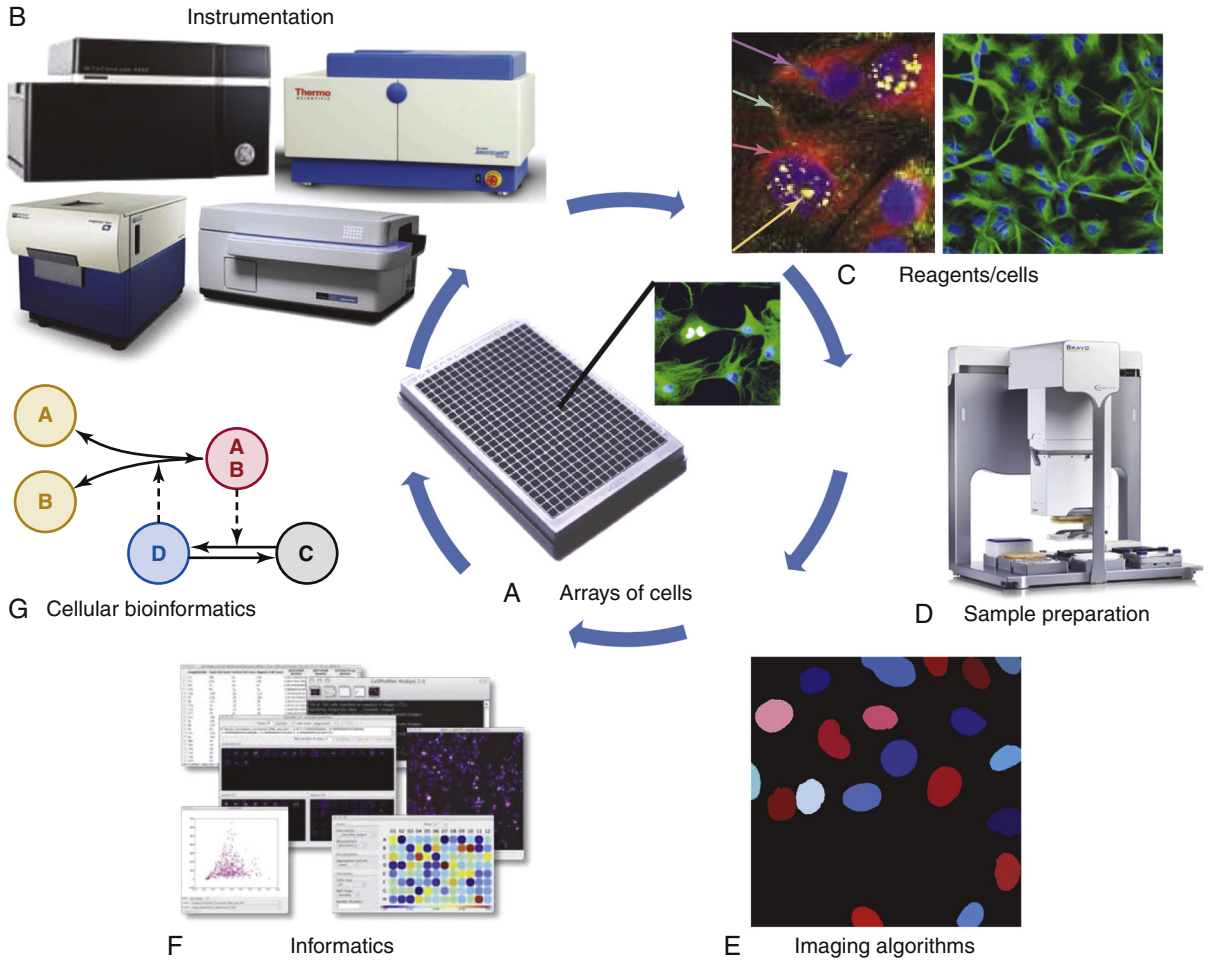


Figure 25-2 The components of high-content analysis (HCA) HCA is defined by the integration of **(A)** arrays of cells/tissues for high-throughput biology; **(B)** automated microscope systems available from multiple vendors; **(C)** a wide range of reagents and cell types; **(D)** automated sample preparation systems and protocols; **(E)** imaging algorithms typically designed to measure multiple features for each cell; **(F)** informatics to review and further process the data, for example, to fit dose-response curves; and **(G)** bioinformatics to relate multiparameter cellular features to biological functions.

Imaging Live Cells and Model Organisms with HCA

Imaging living cells and model organisms by HCA has the advantage of allowing the investigation of the dynamic, temporal-spatial interplay of cellular constituents that define normal and abnormal cell and tissue functions. Single time points and/or kinetic measurements can be generated and analyzed.[20,62] It is also possible to harness advanced, fluorescence-based probes and biosensors to measure physiological parameters not readily measured in fixed samples, such as cyclic protein translocations, pH, free Ca^{2+}, membrane potentials, and a growing number of physiological biosensors by fluorescence microscopy.[63,69] A disadvantage of investigating living systems by HCA is that biological processes can change from the time of imaging the first well in a microplate to the last well and this issue is multiplied when going from 96 to higher-density well plates. Depending on the time course for the specific biological process,[81] including cyclic changes[82] and the protocol for the addition of experimental treatments, large-scale, living samples usually have to be profiled in smaller batch sizes.

All HCA profiling or screening studies start with living systems that receive some combinatorial application of small molecules or biologics,[83-86] RNAi for knockdowns,[87-89] and/or nucleic acids for transfections or transductions. Although more demanding to perform, a recent investigation studied the kinetics of response of individual cells to drug treatments demonstrating the variability of cellular responses in a population.[19] Measuring kinetic responses should increase as even more biosensors are developed and the complex and dynamic aspects of signaling processes are investigated.

Imaging Fixed Cells and Model Organisms with HCA

The main advantage of using fixed samples is that large-scale sample preparation and robotic screening of many microplates or slides is possible without changes in the biology

Table 25-2 Classes of Fluorescence-Based Reagents, Readouts, and Online Resources for HCA

Resource	Type	Application	References
Fluorescent probe classes	Chemical fluorophores	Wide spectral range, easily attached to targeting molecules, some are environmentally sensitive, many useful properties	61, 63, 74, 75
	Nanocrystals	Stable, bright, single excitation, narrow emission, best for multiplexing in fixed cells or cell surface markers	75, 110, 332
	Fluorescent proteins (FPs)	Multiple wavelengths, transient or stable expression, linked to targets, some are environmentally sensitive, photoactivated (or switched), live or fixed cell assays	63, 69
Cellular labeling approaches	Antibodies	Target expression level and localization	63, 69, 75
	FISH probes	DNA copy number variants, RNA expression, including micro-RNA	197
	FPs	Target expression level, localization and dynamics, photobleaching or photoactivation for transport within or between compartments	63, 69
	Environment-sensitive probes	Ion concentrations, membrane potential, hydrophobic compartments	63, 69, 75
	Proximity probes	FRET, colocalization	63, 69, 75, 333, 334
	Enzyme activity	Fluorogenic substrates, cleavable linkers	63, 75
	Organelle specific	Nucleus (DNA), mitochondria, lysosomes, neutral fat, endoplasmic reticulum, etc.	63, 75
	Fluorescent biosensors	FPs or combinations of FPs engineered to report on activation of biomarkers or pathways	63, 69
HCA readouts	Intensity	Relative concentration of target	63, 69
	Distribution	Distribution and dynamics of molecular targets in cells	63, 69
	Colocalization	Similarity or difference in the distribution of two or more labels	63, 69
	FRET	Very sensitive determination of close proximity of two labels	63, 69
	Morphology	Texture, size, or shape of cells or organelles, aggregation	16, 68
	Lifetime	Local chemical environment	63, 69
	Polarization	Molecular interactions (bound vs. free)	63
	Cell tracking	Motility, metastasis	63
	Kinetics	Measure of any or all readouts over time	63, 69
Internet databases	Spectral	PubSpectra, Fluorophore.com, others	335
	Targets	The Human Protein Atlas, The Cell: An Image Library, The BioGRID Interaction Database	336-339
	Cell lines	The Cancer Cell Line Encyclopedia, Cell Line Navigator	133, 340

FISH, Fluorescence in situ hybridization; *FRET*, fluorescence resonance energy transfer; *HCA*, high-content analysis.

Table 25-3 Comparison of Fluorescent and Chromogenic Readouts[177,341-344]

Reporter Type	Advantages	Disadvantages
Fluorescent	Present standard in cell analysis High sensitivity and specificity Quantitative readout Multiplex targets that are colocalized and/or in close proximity Broad spectrum of wavelengths Higher resolution with confocal imaging	Reagents are less stable for long-term storage More expensive fluorescence, more expensive imaging systems More expensive reagents
Chromogenic	Present standard in tissue analysis Long-term stability of labeling Brightfield microscopes Greater amplification	Variable sensitivity and specificity Multiplexed targets must be spatially separated Precipitates cause fuzziness around target

during the readout. Therefore, many combinatorial treatments can be prepared at one time and the microplates/slides stacked in a robotic system for screening/profiling. There are many fluorescence-based reagents including antibodies, fluorescence in situ hybridization (FISH) probes, and fluorescent proteins that can be used to define single time-point localizations, relative concentrations, and activities.[63] In order to optimally interpret fixed samples, either the half-time of a process under investigation must be determined in live sample profiles, or multiple time points must be generated in distinct wells or plates.

HCA has been extensively applied as a phenotypic approach to cancer drug discovery over the past few years, in both primary and secondary screens, either using live-cell[90-93] or fixed-cell[21,39,69,94] screening. Although specific molecular targets guide many of these screens, pathway modulations and phenotypic profiling are central to the approach.[39,71,95,96] Examples of cancer biologies explored include energy metabolism,[97] viral induction,[98] apoptosis,[99] cell cycle,[25,45,91] autophagy,[84] tumor invasion and metastasis,[50] pathway modulations,[47,100] a panel of biologies,[101] and phenotypic changes compared to mutants.[44,102] In many cases, HCA is also used in structure-activity relationship (SAR) to optimize lead compounds.[103,104] However, it is still important to ultimately identify the mechanism(s) of action of lead compounds. The role of HCA in cancer drug discovery and development has been further advanced with the application of more quantitative analyses of profiles using computational biology and systems biology approaches,[105-107] as explored in detail next.

Multiplexed to Hyperplexed Fluorescence-Based HCA

It has been the goal of imaging cytometry to increase the number of specific molecular parameters that can be measured in the same sample, so that complex interplays of components, pathway mapping, and heterogeneity of biological processes can be analyzed in increasing detail. We have defined multiplexed fluorescence in imaging applications as the combination in a sample of up to seven fluorescent probes that can be discriminated by spectral selection. Multiplexing has been accomplished in both live and fixed samples using a range of fluorescent probes.[63,108] Multiplexing by flow cytometry has reached the level of 15 to 18 distinct fluorescent probes,[109] but flow cytometry does not permit analyses of the temporal-spatial dynamics within or between cells. Hence, imaging technologies are being advanced to produce more parameters, especially in fixed samples. There have been a number of technical developments to increase the number of fluorescently labeled antibodies and FISH probes per sample, including new types of probes such as quantum dots,[110] new algorithms such as spectral unmixing,[111] and new protocols such as sequentially labeling, imaging, and quenching the fluorescence, and then repeating the process.[112-115] Recently, the GE Global Research Center has demonstrated that more than 60 fluorescence-based biomarkers can be applied to a single tissue sample using a sequential labeling approach.[111,116] This novel platform technology should have a great impact on basic cancer research, drug discovery, and diagnostics/prognostics. Generating the multiplexed to hyperplexed datasets creates a powerful platform that will enable the application of advanced computational methods to directly define pathways and modifications due to perturbations, as well as to characterize and understand heterogeneity. It is also possible to harness fluorescence lifetime imaging to gain some parameters,[117] as well as the application of mass spectroscopy[118] applied to single cells and tissues, but these latter approaches are not covered here.

In addition, other imaging modalities have been applied to cancer model systems and patients.[33,119,120] The data from these investigations must be integrated into the systems biology models developed in cells, small experimental organisms, and patient tissue sections.

Cellular Systems Biology in Cancer Research and Drug Discovery

Cell Lines, Primary Cells, and Tumor Microenvironments in HCA Studies

The biological interpretation of cancer genomic and proteomic data remains a major challenge.[121] The presence of both genetic and nongenetic heterogeneity among cancer cells complicates population-level data interpretation and implies the need for more detailed analysis at the cellular level.[122-125] Furthermore, the development of diagnostics and therapies requires consideration of the functions and responses in all cancer cells, not just the "average" cancer cell, as well as the role of the microenvironment. There has now been a shift in focus on screening for cancer drugs from relying exclusively on tumor cytotoxicity to understanding the signaling context within which the particular molecular target operates.[126] Multiplexed or hyperplexed cell analysis, when combined with computational modeling, can serve to directly assess multiple functions at the cellular level. Cell-by-cell analysis also allows detailed determinations of heterogeneity in cell populations. Rather than viewing heterogeneity as an interference in the development of diagnostics and therapies, researchers can use computational models of heterogeneity to gain a deeper understanding of the functioning of the underlying pathways and networks, as discussed later.[127] Furthermore, HCA can accelerate mechanism-of-action studies for

oncology research,[128] provided relevant models are used in the analysis. Two important aspects of cancer model development are the choice of cells and the design of the model.

There are now a large number of cancer cell lines available; however, the relevance of those cell lines has long been debated.[129-132] Cancer cell lines are attractive models as they provide an unlimited source of homogeneous, self-replicating material, free of contaminating stromal cells, and the majority are easy to culture in standard media. Although cancer cell lines may have diverged to some extent during culture, the genomics and even pharmacological profiles of large numbers of these cell lines are available and provide a basis for interpreting cellomics profiles in the context of pathways derived from genomics. For example, the recently published *Cancer Cell Line Encyclopedia* (CCLE), a compilation of gene expression, chromosomal copy number, and massively parallel sequencing data from 947 human cancer cell lines, coupled with pharmacological profiles for 24 anticancer drugs across 479 of the cell lines,[133] extends existing data characterizing cancer cell lines.[134-137] Furthermore, results from studying cancer cell lines in two dimensions (2D) serve as the simplest model for comparison with results from more complex 3D models involving tumor microenvironments, as well as results from patient samples, such as those available from *The Cancer Genome Atlas*.[138]

Cell-line fidelity with primary tumors varies depending on the tumor type. For example, the fidelity of breast cancer cell lines with primary tumors, assessed by comparison of genetic heterogeneity and copy number abnormalities, has been shown to be high.[134] Conversely, cell lines derived from glioblastomas have lower fidelity with primary tumors and mostly lack the amplification and mutation of epidermal growth factor receptor (EGFR) that is found in approximately 50% of glioblastomas.[139] Although it is clear that cell lines cannot model the hundreds to thousands of genetic aberrations found in primary tumors, HCA enables sufficient assessment of the many potential aberrations to identify those that are functional.

As a group, breast cancer cell lines faithfully reproduce the heterogeneity in human breast tumors as described earlier, though individually they exhibit profiles that fall short of truly representing the intratumoral heterogeneity of individual breast tumors. Studies suggest that collections of cell lines representing multiple cell types can be used to model the cellular heterogeneity in tissues.[140] Individual cell lines can also be useful models for specific functions. For example, the most commonly used breast cancer cell line, MCF-7, established in 1973 at the Michigan Cancer Foundation, exhibits exquisite hormone sensitivity through expression of estrogen receptor, making it an ideal model to study the hormone response.[141] It is important then to understand how well and which cell lines best model the diversity and which best model specific pathways. Microarray studies have identified molecular subtypes—luminal A, luminal B, ERBB2-associated, basal-like, and normal-like—with characteristic gene expression patterns and underlying DNA copy number alterations (CNAs). Genomic profiling of a collection of breast cancer cell lines found that they retained expression patterns with relevance to the luminal-basal subtype distinctions. That compendium of molecular profiles defines cell lines suitable for investigations of subtype-specific pathobiology, cancer stem cell biology, biomarkers, and therapies and provides a resource for discovery of new breast cancer genes.[142] The choice of cancer cell lines, primary cells, or stem cells to construct models for HCA should be made based on the basis of the abundant genomics, proteomics, and pharmacological profiles of the cell model; the goals of the study; and the analysis methods to be employed.

Whereas 2D cultures of cell lines provide simple models for HCA, pathway and network modeling (see later discussion), and drug discovery,[20,128,143] more sophisticated 3D microenvironment models are required to better recapitulate the tumor environment. 3D cell cultures are rapidly becoming the method of choice for the physiologically relevant modeling of many aspects of nonmalignant and malignant cell behavior ex vivo. 3D models include relatively simple tumor spheroid models[144,145]; more complex extracellular matrix models[146-149]; models that mimic the architecture of specific cancers[150]; cells grown on artificial scaffolds such as engineered organ models[151-154]; and tissue explants.[155,156] In addition, the development of microfluidic devices capable of maintaining a controlled, physiological environment will allow the construction of even more relevant tissue microenvironment models.[157]

In general, the simpler 2D models allow the greatest flexibility in terms of the range of markers and measurements that can be made cell by cell in the monolayer, using multiplexed HCA. Although confocal HCA systems can optically slice through thick specimens, the fact that the cells are not all in the same plane complicates the cell-by-cell quantitation of features. The approaches to the analysis of confocal sections are similar to the analysis of tissue sections, which is discussed in more detail later. The spheroid model is arguably the simplest 3D model for studying tumor cell biology, therapy resistance, cell-cell interactions, invasion, drug penetration, modeling, nutrient gradients, and tumor cell metabolism.[144,145] The self-organization and generation of distinct tumor microenvironments makes it an attractive model for high-throughput imaging.[158] The well-defined geometry of the spheroid simplifies the identification of microenvironments in the model.

There are a wide range of 3D tissue models that can be used with HCA analysis. Extracellular matrix models of

various compositions attempt to reconstruct the in vivo environment for studies of tumor cell biology, cell-cell interactions, cell migration, and invasion. Layers of cells are cultured on top of porous membranes for drug transport and binding, therapy resistance, and invasion assays. Engineered models such as micropatterned surfaces and 3D organizations,[147,150] as well as cells cultured within a network of perfused artificial capillaries for studying tumor cell metabolism, therapy resistance, and artificial organs, are the future. Each of these models, combined with a carefully chosen cell type and analysis approach, can provide insights into many functional components of cancer.[159] A deeper systems biology understanding of cancer will come from integrating data across these studies through the construction of computational models, based on combining pathways based on genomics, with functional data from HCA studies, as discussed later.

Heterogeneity as a Challenge in Cancer Biology and Drug Discovery

Cell-to-cell differences are always present to some degree in any cell population, and therefore the ensemble behavior of a population may not represent the behaviors of any individual cell.[160] Broadly speaking, cellular heterogeneity can be classified as either *genetic* or *nongenetic* in nature.[125] Genetic heterogeneity has been identified as an important factor in cancer progression and is thought by some to result from pressure for cells to adapt to new environments during metastasis or to evade immune responses.[161] Single-cell PCR gene-expression analysis, genomic profiling, immunohistochemistry, and other methods show that cancer tissues contain distinct cell populations and that the different gene expression programs linked to multilineage differentiation are strongly associated with patient survival.[162] Although genetic heterogeneity across cancer lines can be used as a predictor of drug efficacy,[137] intratumor genetic heterogeneity can also impede the development of personalized medicine. Phylogenetic differences, both intratumor and between primary tumors and their metastases, indicate that the sequence of a single sample does not reveal the full complexity of tumor genetics.[2]

Nongenetic heterogeneity is thought to originate from natural biochemical variations between clonal cells, such as differences in concentrations of biological compounds or slight discrepancies in the timing of cellular events. It plays an important role in cancer progression, as subpopulations of cells with significant stable variations in biochemistry exist within primary and metastatic tumor cell populations.[163] Transient drug-resistant phenotypes have been observed to emerge in cancer, conferring drug resistance in the absence of genetic mutation,[164] and drug treatment has even been observed to induce novel phenotypes.[124] The nongenetic heterogeneity in cancer further complicates therapeutic development, as it implies that genetic information by itself may be insufficient to explain the response of a particular tumor to treatment, even if distal regions of the tumor are sequenced. An emerging course of action is to address cancer at the level of pathway, simultaneously targeting multiple pathways to minimize potential drug resistance. In addition, defining the immune status and stromal cell content in tumors is critical to understanding individual responses to therapeutics.

Analyzing single cells within a population is a new frontier in platform technologies that has the potential to transform systems biology through new discoveries derived from cellular heterogeneity.[127] A great amount of information about the biochemical and environmental conditions of genetically homogeneous cellular populations can be uncovered by exploiting the differences between individual cells within the population. Patterns of heterogeneity in basal populations have been used to predict drug sensitivity in clonal populations of the NCI-60 panel of cancer cell lines,[165] and phenotype profiling has been applied to uncover compound mechanisms of action.[166-168]

The general approach to extracting biochemical information from inherent heterogeneity commences with the identification of well-defined phenotypes, through either expert opinion or automated clustering algorithms.[106] In the case of the former, supervised machine learning techniques are used to teach a computer how to correctly recognize phenotypes and classify cells. It has been shown that a computer trained by an experienced biologist to recognize 14 distinct morphological phenotypes can accurately classify human cancer cells at a rate of 70,000 per second.[169] Alternatively, unsupervised learning methods can be employed to allow computational identification of phenotypes based on clustering in feature space, without prior human interpretation. As an example, cellular phenotypes that were automatically identified using a support vector machine have been used to extract information about drug activity in HeLa cells.[105] In either case, it is necessary to use a set of features that is capable of distinguishing between phenotypes.[170] Once phenotypes are established and recognizable by computer, entire cell populations can be described in terms of the relative abundances of their constituent phenotypes.[79,171] *Statistical* or *mechanistic* techniques are used for analysis of the patterns of heterogeneity. Statistical analysis techniques involve comparing phenotype distributions that result from novel treatments to those that are associated with treatments of known action. Standard statistical measures, such as the KS statistic[172] or KL divergence,[173] are used to compare population distributions, and predictions for novel treatments

are based on which known treatments they most closely resemble. In mechanistic analysis, one or more cellular pathways of interest are modeled in silico, and their predictions are compared to the patterns of heterogeneity in the population. Predictions of concentrations of biomolecules within cells from pathway models can be compared directly to measurements from HCA experiments, and multiple pathway models can be combined to computationally represent a heterogeneous population of cells.[127]

Figure 25-3, *A*, illustrates the automated clustering of Cal33 head and neck cancer cells based on the relative activation of STAT1 and STAT3 in response to treatment with IFNγ and IL6 as measured by phosphorylation or translocation to the nucleus. In many cancers, abnormal activation of STAT3 functions to promote tumor growth, whereas STAT1, despite a greater than 50% sequence homology, is mostly antagonistic to STAT3 activity.[174] A better understanding of the differences in regulation and activation of STAT1 versus STAT3, on a cell-by-cell basis, will be important for therapeutic development. The cells were clustered on the STAT1 and STAT3 activation state using a Gaussian mixture model. The fraction of cells in each cluster varies over time, with a rapid but transient activation of STAT3 followed by a slower but more sustained activation of STAT1 (see Figure 25-3, *B*). The extent of each cluster indicates a highly heterogeneous level of activation, and the inclusion of a fraction near the origin indicates cells that appear to be unresponsive. Whether this is a kinetic effect or the result of the cells being in an activation-resistant state is still under investigation.

Tissue Systems Biology in Cancer Diagnostics/Prognostics

From H&E Staining to Multiplexed and Hyperplexed Fluorescence

For decades pathologists have used hematoxylin and eosin (H&E) and immunohistochemistry (IHC) with manual analysis by microscopy to assess tissue morphology and protein expression and distributions for diagnostic purposes. Although these methods are very valuable and are still used in the majority of cancer diagnoses, they are limited by subjectivity and intra- and interobserver variability, and they are only semiquantitative (see Table 25-3). There is a growing trend to digitize pathology to improve objectivity, standardization, and productivity, as has been done in radiology. Brightfield and fluorescence digital slide scanners from companies such as Aperio Technologies,[175] BioImagene (now Ventana Medical Systems),[176] Perkin Elmer, Omnyx, and 3DHistech[177] are increasingly being adopted in medical laboratories. Slide scanners produce whole-slide digital images for viewing and sharing by telepathology and to enable more reproducible analysis of morphology and biomarkers by image analysis software. Digital image analysis has been applied to IHC-labeled biomarker analysis in multiple types of cancers to improve reproducibility and standardize scoring methods.[178-181] Even with digital slide analysis, the accuracy of chromogenic IHC is limited by the inherent staining variability associated with the nonlinear signal amplification and is mostly limited to a few biomarkers

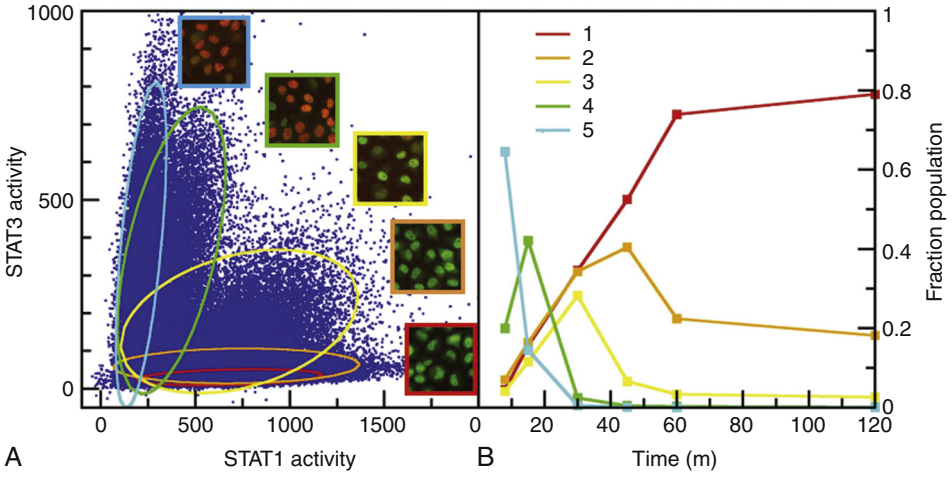


Figure 25-3 Automated identification and quantitation of subpopulations of cells **(A)** The scatterplot illustrates the relationship between STAT3 and STAT1 activity for ~72,000 Cal33 head and neck cancer cells stimulated with 50 ng/mL of IL-6 and 50 ng/mL of IFNγ for 8-120 min. STAT3 activity measured as fluorescence intensity of an antibody to phospho-STAT3-Y705, and STAT1 activity measured as the nuclear-cytoplasmic difference in fluorescence of an antibody to STAT1. Five subpopulations (*blue*, *green*, *yellow*, *orange*, and *red ellipses*) were automatically identified by a Gaussian mixture model algorithm. Pseudocolor images *(inset)*, STAT1 (*green* channel), and STAT3 (*red* channel) show the variation in labeling seen among the five clusters. **(B)** The distribution of the cells between subpopulations changes over time (*colored lines* correspond to clusters). At the earliest time point, 8 min, over 60% of the cells are in cluster 5 *(blue)*, principally STAT1(–) and STAT3(+). At 15 min, the largest fraction has shifted to cluster 4 *(green)* showing a slight activation of STAT1 and already a decrease in the STAT3 maximum. At 30 min, the cells are about equally distributed among clusters 3 *(yellow)*, 2 *(orange)*, and 1 *(red)*. By 1 hour, clusters 1 and 2 account for >95% of all the cells.

per slide (see Table 25-3).[182,183] Multiplexed IHC can be performed,[184] yet multichromagen images are more difficult to interpret visually and to deconvolve with image analysis software, particularly for biomarkers expressed in the same subcellular compartment (see Table 25-3). However, there is now some promise in applying unsupervised learning methods to chromagen-labeled samples.

Quantitative, multiplexed digital imaging methods can improve reproducibility and also measure the multitude of molecular changes in cancer that are associated with disease subtype, tumor progression, and response to therapies. Because these molecular changes precede morphological changes in the development and progression of cancer, molecular profiling can improve the sensitivity of diagnostic and prognostic testing in conjunction with histopathology.[185] Cancer progression and responses to therapies depend on multiple components of many signaling pathways within malignant cells and stromal processes. It has become clear that there are no "single bullet" biomarkers and that multiple biomarkers are required to accurately diagnose, predict risk of recurrence, and/or predict response to therapies for all patients with a particular type of cancer. Multiplexed fluorescence biomarker labeling with digital imaging represents a significant improvement over traditional histologic methods for tissue biomarker analysis, including the ability to quantify multiple antigens per tissue section, the ability to quantify antigens that are colocalized to the same subcellular compartment, and more consistent, linear, higher resolution labeling with greater dynamic range of biomarker measurements. Multiplexed biomarker analysis in the context of tissue morphology builds on cellular HCA, described earlier, and allows objective, reproducible extraction of quantitative biomarker data and morphology data by image analysis software.[186-189] Quantitative multiplexed fluorescence analysis of biomarkers in digital tissue images has been used to measure diagnostic, prognostic, and predictive cancer biomarkers in multiple cancer types.[102,186,190,191] These methods of protein biomarker measurement correlate with Western blot analysis,[192] match or exceed the accuracy of manual IHC scoring,[193-195] and can improve standardization of tissue biomarker measurements.[196] Labeling and imaging of nucleic acids in tissues can also add power to cancer testing. Locus-specific amplifications and losses and also microRNAs can be measured by FISH in intact tissues to enable interpretation within the context of tissue architecture.[197]

Multispectral imaging enables separation of overlapping dyes in both brightfield and fluorescence tissue images to increase the potential level of multiplexing.[177] Nanoparticles such as quantum dots and composite organic-inorganic nanoparticles (COINs) have emission spectra that are narrower and more symmetrical than traditional fluorophores, which results in minimal crosstalk between fluorescence channels and offers the potential for higher levels of multiplexing in tissues.[110,198] Hyperplexed fluorescence methods, defined earlier, can overcome the spectral limitations of fluorescence-based detection of biomarkers and offer the potential to assess dozens of protein and nucleic acid parameters per tissue section for protein network topology, spatial mapping of protein clusters, microRNAs, and CNVs in tissues.[114-116,199] Infrared spectroscopic imaging and Raman scattering imaging of unlabeled tissue sections enables the collection of thousands of spectra representing biochemistry in the context of the tissue architecture.[200,201] This type of high-content tissue imaging can be used to classify cancer types and distinguish cancer stages[202-204]; however, the mechanisms underlying the classifications are difficult to interpret because the specific molecules responsible for the hyperspectral signatures are not known.

Heterogeneity and Complexity of Tumors: Breast Cancer as an Example

Tumor heterogeneity and complexity highlight the need for a "tissue systems biology" approach to cancer diagnostics, as described in the first section and as exemplified by breast cancer. The current standard diagnostic and predictive tests for breast cancer are IHC-based measurements of estrogen receptor (ER), progesterone receptor (PR), and HER2/neu.[205] These biomarkers are usually measured one at a time in serial sections with manual scoring by a pathologist. HER2/neu status is confirmed by FISH in a subset of patients who are candidates for HER2/neu-directed therapies. Multiplexed fluorescence immunohistochemistry has been applied to these standard breast cancer biomarkers to improve standardization.[206,207] This limited set of biomarkers is insufficient to address breast cancer heterogeneity and cannot accurately stratify patients according to breast cancer subtype, predict risk for recurrence, or predict benefit from therapies. Gene expression profiling has identified molecular portraits of the main breast cancer subtypes and characterized the genetic and epigenetic abnormalities associated with each subtype.[1,3] RT-PCR– and DNA microarray–based multigene tests have been developed for prognostic and predictive testing in breast cancer. Oncotype Dx (Genomic Health, Inc., CA, USA) and Mammaprint (Agendia, BV, Netherlands) are two such tests that have achieved clinical adoption.[208-212] These approaches are valuable and have had a positive impact on patient care; however, the various multigene tests for cancer can be limited by bias introduced by interpatient variations in percentages of malignant, immune, and stromal cells. Furthermore, these methods require tissues to be digested, and the tissue architecture and spatial information, which are important for accurate biomarker

Table 25-4 Examples of Cancer Biomarkers Exhibiting Subcellular Relocation Based on Pathway Activity

Biomarker	Subcellular Relocation Based on Pathway Activity	References
NFκB	Restricted to cytoplasm in resting state, translocates to the nucleus on activation, and induces transcription of genes involved in inflammation, proliferation, and apoptosis in malignant cells and stromal cells.	345, 346
Beta-catenin	Activation of Wnt signaling leads to nuclear translocation of beta-catenin, which drives epithelial-mesenchymal transition and metastasis.	347-349
STAT1, STAT3, STAT5	Activation of STATs requires phosphorylation and nuclear translocation, which are associated with cancer prognosis.	215, 350, 351
p21	Nuclear localization is correlated with the inhibitory effect of p21 on cancer cell growth, whereas cytoplasmic localization is associated with protection from apoptosis.	352-354
HIF1-alpha	Master regulator of oxygen homeostasis that is cytoplasmic under normoxic conditions, translocated to the nucleus in response to hypoxia. Associated with clinical outcome in multiple cancer types.	355, 356
FOXO3a	Tumor suppressor that is active when localized to the nucleus and inactive when in the cytoplasm. Nuclear localization is associated with good prognosis and cytoplasmic localization is associated with poor prognosis in breast cancer.	357

measurement and interpretation, are lost. Stromal signatures including immune response and angiogenesis-related genes have been shown not only to add prognostic information but to have improved prognostic significance over standard breast cancer biomarkers and whole-tumor signatures in breast cancer.[213] The rationale for profiling biomarkers by digital imaging in intact tissue sections is based on extensive literature describing the importance of protein level, activation status, subcellular localization, tissue localization, spatial relationships, and distributions for the accurate measurement and interpretation of cancer biomarkers. Biomarker function is often more relevant to clinical variables than overall expression. For example, transcription factors such as nuclear factor-κB (NFκB) and signal transducers and activators of transcription (STATs) are ubiquitously expressed but are frequently activated in breast cancer.[214-216] Their activation is measured by translocation to the nucleus and/or by phosphorylation. Examples of cancer biomarkers exhibiting subcellular relocation based on pathway activity are provided in Table 25-4,* and tissue images showing nuclear translocation of transcription factors are shown in Figure 25-4.

Spatial relationships and microenvironments within the tumor system are also important in breast cancer. A three-part arrangement of an invasive breast cancer cell, a macrophage, and an endothelial cell in breast cancer has been described as a microenvironment conducive to distant metastasis.[217] The combined density and spatial distribution of mature dendritic cells and activated T cells has also been shown to have prognostic significance in breast cancer.[218,219] Macrophages infiltrate only the stroma in subsets of breast

*Table 25-4 contains citations of References 215 and 345 through 357.

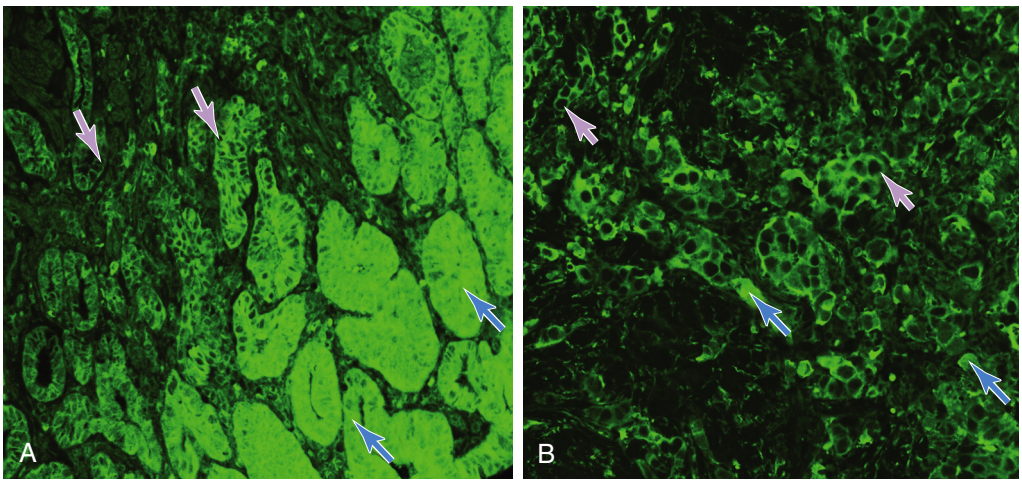


Figure 25-4 Multiplexed immunofluorescence labeling and digital imaging of functional biomarkers in tissues Sections of esophageal tissue with intestinal metaplasia **(A)** and breast cancer tissue **(B)** were labeled with a primary antibody specific to NFκB p65, fluorescently labeled secondary antibody, and Hoechst 33342 (nuclear stain). Slides were imaged using a fluorescence digital slide scanner (Aperio FL, Aperio Technologies, Inc., Vista, CA) at 20× magnification. Examples of cells exhibiting resting state NFκB in the cytoplasm are highlighted with *purple arrows* and cells with activated nuclear NFκB with *blue arrows* (**A** and **B**).

cancer patients and infiltrate the tumor nests in other patients (Figure 25-5, G, *H*), which has been correlated with survival in breast cancer patients.[220] This valuable spatial information is lost in molecular profiling approaches that digest tissues to extract DNA, RNA, or proteins, but is preserved and can be quantified by digital imaging approaches. Digital imaging of intact tissues also enables measurement of nuclear morphometric features and the amount of DNA in tumor cells, which have diagnostic and prognostic significance in breast cancer.[221] Multiplexed-to-hyperplexed biomarker imaging is also important for the measurement of specific cell types and phenotypes such as cancer stem cells[222,223] and immune cell subsets[224] that require multiple biomarkers for accurate classification. A major goal of hyperplexing will be to identify the optimal combination of protein, DNA, and RNA biomarkers that can stratify the disease subpopulations. Subsequently, a multiplexed subset of the biomarkers will be used in the development of diagnostic/prognostic tests. Figure 25-5 illustrates multiplexed immunofluorescence imaging of serial sections of a breast cancer tissue microarray, scatterplot analysis of the relationships between single cell features derived from the images, and examples of immune cell subsets infiltrating the cancer stroma and tumor cell nests.

Integration of Digital Imaging with Other Datasets

Multiregion spatial DNA sequencing has revealed significant intratumoral genetic heterogeneity that is underestimated by sequencing single tumor biopsies.[2] Quantitative pathology with digital imaging of intact tissues can be coupled to other platform technologies to allow HCA of various macromolecules and whole-genome sequencing in specific tumor microenvironments and cell populations to fully assess intratumoral heterogeneity. Tumor microenvironments and cell populations can be identified by digital imaging and captured by laser capture microscopy (LCM) or by coring of tumor samples for further genomic and proteomic analysis.[225-227] Sequencing of specific regions or the whole genome can be performed on the purified cell populations.[225] Proteomic profiling by mass spectrometry has been applied to highly enriched captured cell populations to characterize breast tumor microenvironments and to elucidate specific regulatory pathways involved in breast tumorigenesis.[226,228] Profiling of mutations in captured cell populations has been used to characterize genetic heterogeneity and to aid cancer diagnostic testing.[227,229] Gene expression profiling has also been applied to LCM-enriched epithelial cell populations to identify signaling pathways associated with specific subtypes of breast cancer[230,231] and to elucidate signatures associated with epithelial and stromal compartments that have diagnostic and prognostic significance.[213,232] DNA methylation analysis in purified cell populations from tumors has revealed changes in methylation of genes in specific cell types within the tumor system.[233,234]

The integration of the foregoing data sources with digital imaging technology and machine learning can be accomplished by employing a cellular/tissue systems biology approach like that illustrated in Figure 25-6. Genomics and proteomics have inferred pathway maps associated with normal tissue and alterations of those pathways associated with diseased tissue. Pathway maps provide insights into the composition and topology of the signaling network. However, they cannot be used to determine activation states of key proteins in a pathway, they do not provide quantitative information on relationships between pathways, and they do not provide information about cell-to-cell variability.[235] Multiparameter imaging of tissues and dynamics of cellular models, along with other platform data, combined with machine learning enables the identification and characterization of the dynamics and heterogeneity in signaling pathways, and the construction of computational models that provide a deeper understanding of the normal and abnormal functioning of those pathways.

Tools to Address Heterogeneity in Tissue Systems

Digital imaging of multiplexed biomarkers in tissues enables assessment of both genetic and nongenetic heterogeneity in tumor tissue systems. Quantitative multivariate data can be extracted from digital tissue images of protein, DNA, and RNA biomarkers at single-cell, subcellular, and tissue compartment levels, and spatial relationships can be measured. The digital imaging challenge is to separate neighboring and overlapping nuclei to enable single-cell and subcellular analyses. Approaches such as the watershed algorithm use grayscale information to separate nuclei by finding valleys of low intensity between the high intensities of nuclei centers. A priori knowledge about the shape, size, and intensity distribution of fluorescently labeled nuclei can also be used with pattern recognition algorithms, to guide image analysis algorithms and improve nuclear segmentation.[236-238] Once nuclei are segmented, the cytoplasm and plasma membranes of each cell can either be estimated based on the nuclear masks or specifically masked using cytoplasmic- and plasma membrane–restricted biomarkers. There are many commercially available image analysis software packages from companies such as Definiens, AG (Munich, Germany),[239] HistoRx (Branford, Conn, USA),[196] and Hamamatsu Photonics, K.K. (Hamamatsu City, Japan)[186] that can segment nuclei and enable

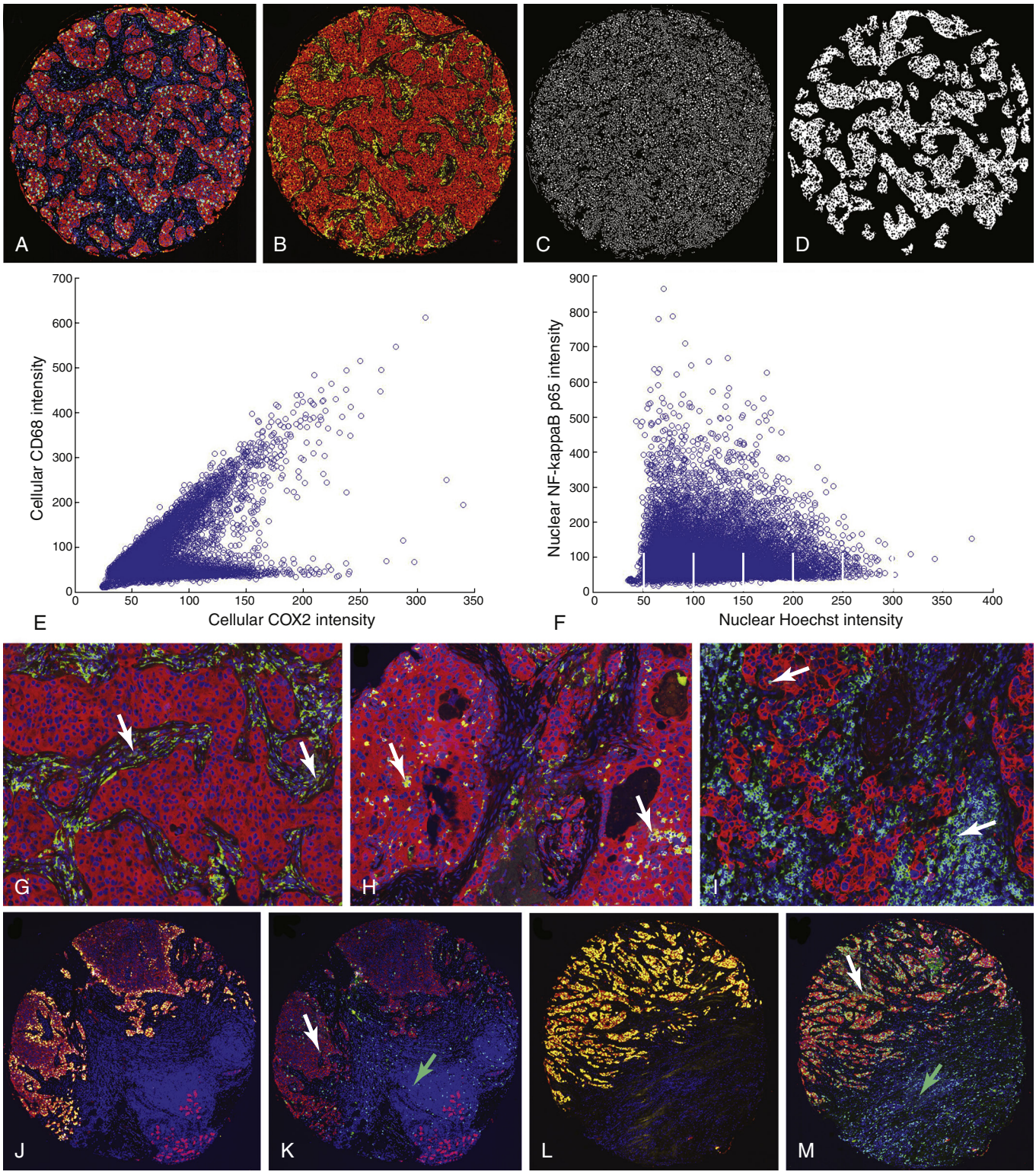


Figure 25-5 Multiplexed immunofluorescence and quantitative digital image analysis of tumor microenvironment biomarkers in breast cancer Serial sections of a breast cancer tissue microarray were labeled with primary antibodies specific for Ki-67, pan-cytokeratin, CD68, COX-2, NFκB p65, PD-1, and CD45RO, fluorescently labeled secondary antibodies specific to each primary antibody, and Hoechst 33342 (nuclear stain). Slides were imaged using a fluorescence digital slide scanner (Aperio FL, Aperio Technologies, Inc., Vista, CA) at 20× magnification. The digital images were analyzed using TissueCipher software (Cernostics, Inc., Pittsburgh, Pa) to segment nuclei, cells, and tumor cell nests as individual objects in which quantitative biomarker intensity measurements were made. **(A)** Ki-67 (*green* nuclear signal in both stroma and tumor nests), pan-cytokeratin (*red* plasma membrane signal in tumor nests) and DNA (*blue* nuclear signal) in breast cancer tissue core. **(B)** CD68 (*green* plasma membrane signal in stroma), NFκB p65 (*red* cytoplasmic and nuclear signal throughout tissue), COX-2 (*yellow* plasma membrane and cytoplasmic signal throughout tissue), and DNA (*blue* nuclear signal). **(C)** Nuclear image analysis mask. **(D)** Tumor nest image analysis mask. **(E)** Single cell measurements of cellular CD68 and COX-2 in the breast cancer tissue shown in image **B**. The bivariate data show that the tissue is composed of COX-2–positive, CD68-negative malignant cells and CD68-positive cells, i.e., macrophages, with a subpopulation expressing high levels of COX-2, which are known to promote tumor angiogenesis. **(F)** Single cell measurements of nuclear NFκB p65 and DNA in the breast cancer tissue shown in **B**, which shows a subpopulation of cells with nuclear localization of NFκB p65, promotes breast cancer cell migration and metastasis. **(G,H)** CD68 (*green* as in **A**), NFκB p65 (*red* as in **B**), and DNA *(blue)* showing stroma-restricted infiltration of $CD68^+$ macrophages **(G)** and macrophages infiltrating tumor nests **(H)** (examples indicated by *arrows*). **(I)** PD-1–expressing tumor cells (*red* plasma membrane signal) surrounded by $CD45RO^+$ memory lymphocytes (*green* plasma membrane signal) in the stroma and tumor nests (examples indicated by *arrows*); **(J-M)** p53 (*yellow* nuclear signal), Ki-67 (*green* nuclear signal), pan-cytokeratin (*red* plasma membrane signal) showing heterogeneity in p53 overexpression within tumor nests **(J)** and proliferating tumor cells and stromal immune cells, indicated by *white* and *green arrows*, respectively.

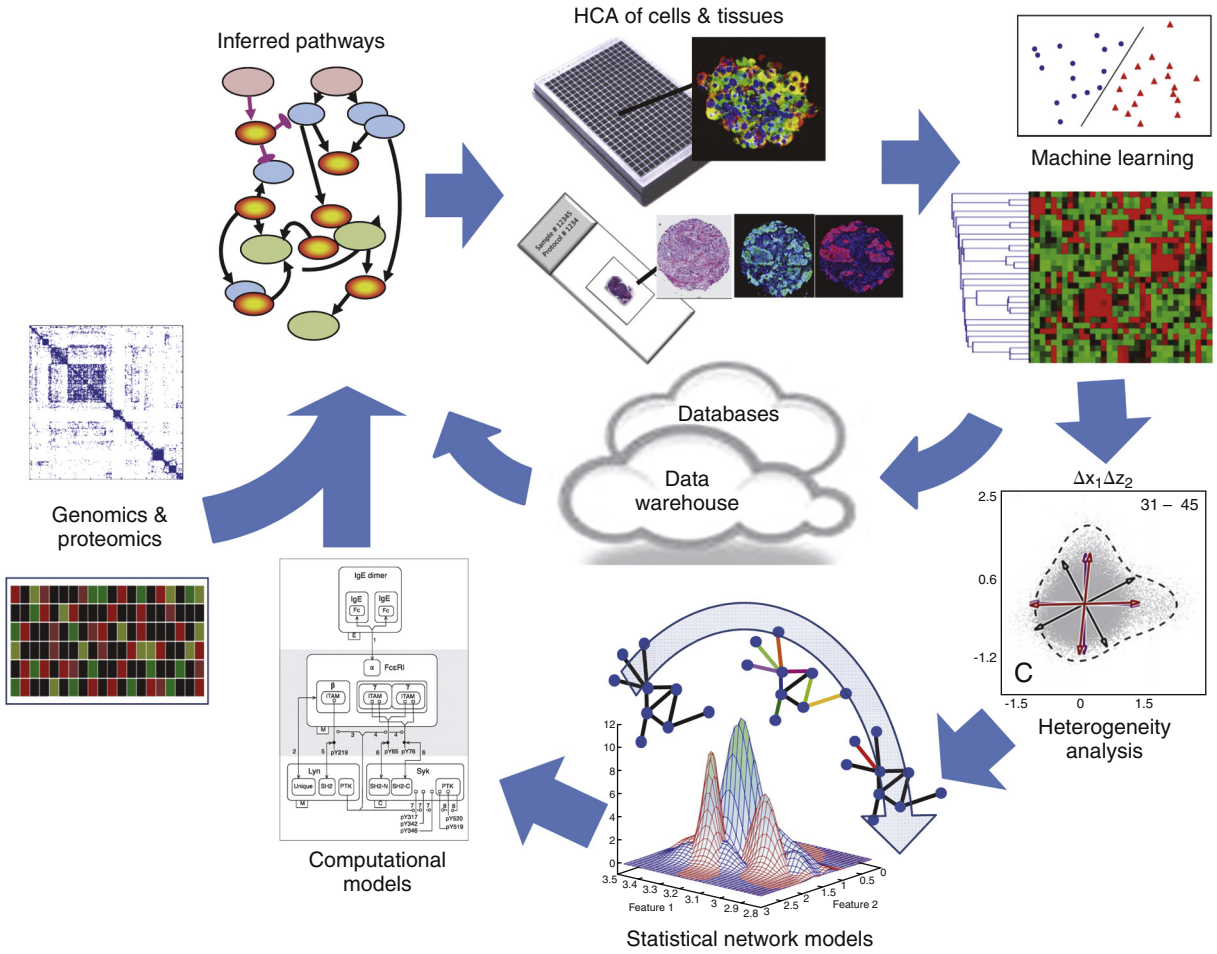


Figure 25-6 Application of cellular/tissue systems biology Cellular signaling pathway maps inferred from gene and protein expression data are used to select multiple biomarkers for interrogation in cells and tissues. The multiparameter data are used to identify correlations, construct classifiers that predict physiological conditions, and analyze cellular heterogeneity. Heterogeneity can be modeled as a statistical combination of networks that are used to refine computational models, to update the pathway models, and to select additional biomarkers for HCA.

quantitative measurements of biomarkers in subcellular compartments and measure tissue morphometric features.

Variation in tumor microenvironment is thought to be an important driver of tumor cell heterogeneity, as well as an impediment to treatment.[240] In order to understand the relationship between cellular heterogeneity and microenvironment, it is necessary to characterize the local environment of each cell in addition to the biomarker activities. Figure 25-7 illustrates an algorithm designed to identify and classify tissue regions with similar microenvironments. The image is divided into a series of small patches (see Figure 25-7, *A*). A feature set that characterizes biomarker intensity distributions within and between cells is measured for each patch. The similarity matrix (see Figure 25-7, *B*) indicates the degree of similarity between patches. The similarity matrix is converted to a network (see Figure 25-7, *C*) that is used to cluster the patches into groups with distinct microenvironments.[241-243] The correlation of biomarker activity with microenvironment class will be useful for the identification of biomarkers that are more commonly affected by the local environment and aid in the interpretation of the heterogeneity exhibited by those biomarkers.

Although computational imaging provides quantitative information on the structure and function of cells and subcellular structures, there are several key challenges in addressing tissue heterogeneity in digital tissue images. Robust, scalable image analysis tools are required that can operate in extremely low signal-to-noise ratio regimes and handle the data-intensive challenges presented by multiplexed and hyperplexed tissue biomarkers. Computational approaches must carefully analyze the long-tail behavior of biomarker intensity and feature distributions in order to characterize heterogeneous subpopulations of cells. Furthermore, the reasoning strategies of pathologists need to be incorporated into analysis approaches to flag diagnostically relevant areas of tissue images for cancer detection and analysis. These challenges can be addressed by machine learning approaches, which enable expert domain knowledge and rule-based decision making to be incorporated to guide tissue image analysis.

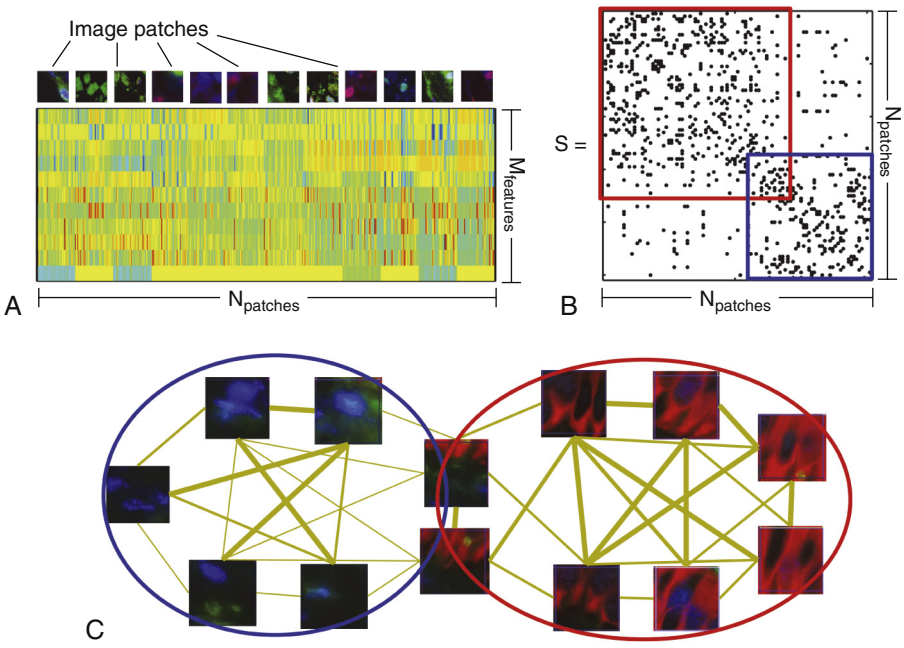


Figure 25-7 Automated identification of cancer cell microenvironments
(A) A fluorescent image is broken into small homogeneous texture regions ("patches") and a feature vector is measured for each image patch (e.g., distributions of biomarker intensities on nuclei, cells, and cytoplasm). **(B)** Matrix S indicates the degree of similarity between each texture patch. Nonzero elements of matrix S are indicated by black dots. **(C)** The similarity matrix can be converted into a network, so that each image patch is a node in the network, and values in S describe weights on edges between image patches (shown by the thickness of the connecting edge). By performing a random walk on the network, identifying bottlenecks, and removing them, a clustering of the nodes into groups of homogeneous image patches emerges naturally—each cluster containing a distinct microenvironment (shown in *colored ovals* in **C**).

Computational and Systems Biology Methods in Cancer Research and Diagnostics

Machine Learning and Other Computational Methods in Cancer Research, Drug Discovery, and Diagnostics

In parallel to the dramatic expansion of automated instrumentation for collecting biomedical research data over the past 30 years, machine learning has arisen as a powerful approach to the analysis and interpretation of this and many other kinds of data.[244] The field of machine learning has already led to many revolutionary technologies, from autonomous vehicles to voice commands to question-answering systems such as Watson, and promises much more. There are two distinct paradigms for posing questions using machine learning, with many intermediate approaches that combine them. These are *supervised learning*, or learning by example, and *unsupervised learning*, or learning models from data. In both approaches, a large set of objects/observations are provided that are described by various *features*. Objects can include individual cells or patients and features are measures such as cell size or blood antigen levels.

Within supervised learning there are two types of challenges: (1) *classification*, i.e., recognition of specific object classes, and (2) *regression* to estimate the value of output variables. In both supervised problems, the class names or output variable values for some of the objects form a *training set*, and the task is to learn a rule or rules that allow the class/value to be predicted/estimated from the feature values for new objects. Accuracy of the system is measured using a *test set*, or the training set can be divided into portions and some portions used for training and others for testing.

There are many frameworks for supervised learning, ranging from simple linear approaches (linear discriminants, nearest neighbor classifiers), to piecewise linear (decision trees), to highly nonlinear (artificial neural networks, support vector machines with nonlinear kernels). All have been increasingly used in biomedical research, particularly in analyzing results from microarray experiments. For example, gene expression profiles have been used to distinguish tumor samples from normal tissue.[245,246] Similar approaches have been used to correlate serum protein profiles measured by mass spectrometry with the presence of particular cancer types in patients.[247] Classification of gene expression data can also provide information for determining treatment or prognosis. This approach has been used to distinguish the tissue site of tumor origin[248] and to distinguish metastatic from nonmetastatic tumors.[249]

Similar approaches can be used to analyze biomedical images, in which the most challenging task is to decide on, and calculate, the features that describe a particular image or region of an image. Magnetic resonance imaging (MRI) is widely used for detection and staging of tumors, and performance on these tasks can be improved using machine learning.[250] Supervised learning methods have also been applied to analysis of traditional histopathology and have achieved similar, and often superior, results to manual scoring by pathologists.[251] These approaches use intrinsic tissue structural information; however, additional information can be gained by analyzing molecular changes in cancer tissues, such as gene expression levels or subcellular localizations of proteins.[252-254]

In unsupervised learning, only feature values are provided. Analogous to classification and regression in supervised learning, the challenge is either to discover classes or clusters that are present, or to build a model that allows estimation of some of the features from the others. The principle behind cluster analysis is to group observations based on similarity in feature values as measured by a distance function. Cluster analysis has been widely used to find clusters of genes with similar changes in expression level in response to some stimulus.[255] A variation on this theme is to perform cluster analysis and then validate the clusters by finding other distinguishing characteristics. For example, patients have been grouped into clusters based on DNA microarray data, and those clusters were shown to differ in clinical phenotypes.[256] In HCA, cluster analysis can be used to discover patterns that are present in a collection of images, whether for learning protein localization patterns[257] or for grouping compounds by their effects on cells.[21,106] In addition to characterizing average phenotypes across a population of cells, heterogeneity of responses can be determined automatically by finding clusters of cells that differ in their multivariate phenotype.[258] This approach has been demonstrated to be useful for predicting the response of various tumor cell lines to chemotherapeutic agents.[165]

Although most machine learning applications use the basic paradigms of supervised and unsupervised learning, there are many intermediate approaches termed *semisupervised learning* that use a degree of supervision to accomplish unsupervised learning goals. For example, unlabeled data can be used to learn probability distributions for features, and this knowledge can improve classifier training. Alternatively, labeled data can be used to learn a distance function that is then used for unsupervised learning. Human intervention can also be used to tune distance functions or classifiers; this is termed *interactive supervision*. An initial clustering can be done using all of the data without labels, and the distance function parameters can be optimized based on user feedback. Alternatively, examples of two or more classes can be given with classes assigned for unlabeled data, and then corrections can be made or new classes defined.

Interactive semisupervised learning can be a form of what is termed *active* machine learning. A semisupervised learning system would be termed active if the system chooses which points the human should focus on rather than displaying all points and allowing the human to choose points. Most machine learning methods assume that all of the data, i.e., features and/or labels, for a problem have already been obtained. Active learning deals with situations in which the data are incomplete but where it is possible to acquire more. Active learning begins by building a predictive model from currently available data using one or more of the methods described earlier. Various methods are then used to decide which missing data to acquire, with the goal of maximally improving the model. Active learning approaches are likely to be highly relevant to HCA as part of drug development.[80] Selection of drug candidates must be guided both by the desired effect on a given target and by lack of effects on other cellular targets that may lead to side effects. Given that the number of potential targets is over 10,000, and that the number of potential drugs is at least 1 million, screening all combinations would be prohibitively expensive. An alternative is to assay a representative subset of targets thought to reflect major cellular pathways.[259] This is clearly an improvement over current practice, but the complexity and interconnection of those pathways present significant challenges.

Two algorithmic approaches, namely *robust statistics*[260] and *spectral graph-theory*,[261] from the fields of computer vision and machine learning have the potential to address emerging challenges in digital tissue biomarker analysis. Robust statistics provide a suite of algorithms[262] to (1) fit parametric models to noisy data, e.g., to model a membrane wall with a parametric shape and extract the parameters of the shape model from noisy images of a membrane wall; (2) instantiate multiple occurrences of a model whose number and type must be determined from the data; and (3) account for structured outliers, i.e., discount certain observations in the data so that they do not affect the estimation of the model parameters. Algorithms from robust statistics and spectral clustering have significantly advanced the state of the art in the fields of computer vision and machine learning. These approaches are expected to be highly relevant to and widely applicable to the computational tasks of analyzing digital pathology datasets.[263-265]

Computational Modeling of Cellular Systems

The heterogeneity and complexity of cancer also pose major challenges for computational modeling. Cancer has signatures and mechanisms that operate at multiple scales. Two fundamental scales that are bridged by 2D and 3D cellomic data are the intracellular molecular network scale and the cellular scale at which cell-cell interactions take place. Just as experimental models must go beyond the uniformity of a single cell line, computational models at the molecular level must consider far more than single networks in isolation if they are to provide a realistic description. At the same time, models of processes at the cell population level must incorporate molecular details if they are to reveal underlying mechanisms through which cancer mutations and other disease-associated changes exert their effects. Bridging between the molecular and cell population levels is a major challenge for computational modeling going forward. Achievement of this integration will lead to improved mechanistic understanding of cancer and holds promise for the development of new therapies.

In a mechanistic model, cellular activity is represented as a directed network of chemical interactions. Each node of the network represents a type of molecule in or around the cell, and each network edge represents a chemical interaction. The system's behavior is determined by the network topology, molecular concentrations, and reaction constants, as illustrated in Figure 25-8.

The first major task in computational modeling is to identify a model or set of models that is compatible with both prior knowledge about underlying molecular and cellular mechanisms and the available data. In the discussion below we focus on the specific requirements for developing models based on HCA data, but the procedures for developing models are quite general. Figure 25-9 illustrates the

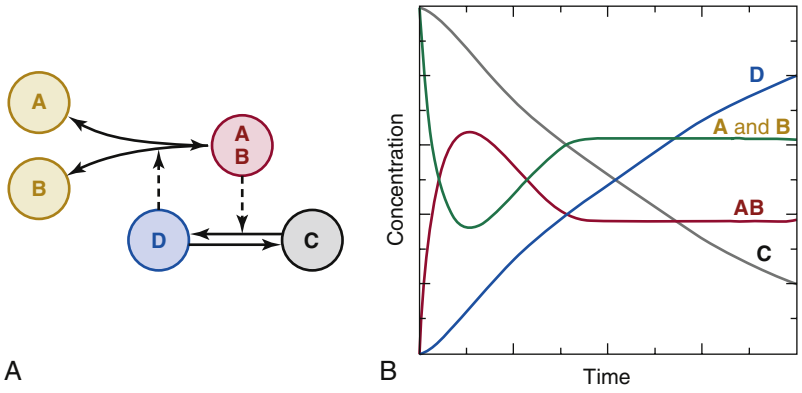


Figure 25-8 A simple reaction network and its output as an example of mechanistic pathway modeling **(A)** Reactants A and B *(green)* reversibly combine in equal amounts to form the homodimer AB *(red)*, which catalyzes the conversion of C to D *(blue)*. D in turn catalyzes the dissociation of AB into A and B. The system of equations represented by this network can be solved to find how the concentrations evolve in time. **(B)** The time evolution of molecular concentrations if A, B, and C are initially present in equal amounts and AB and D are initially absent. The shapes of the curves depend on the rates of the chemical reactions.

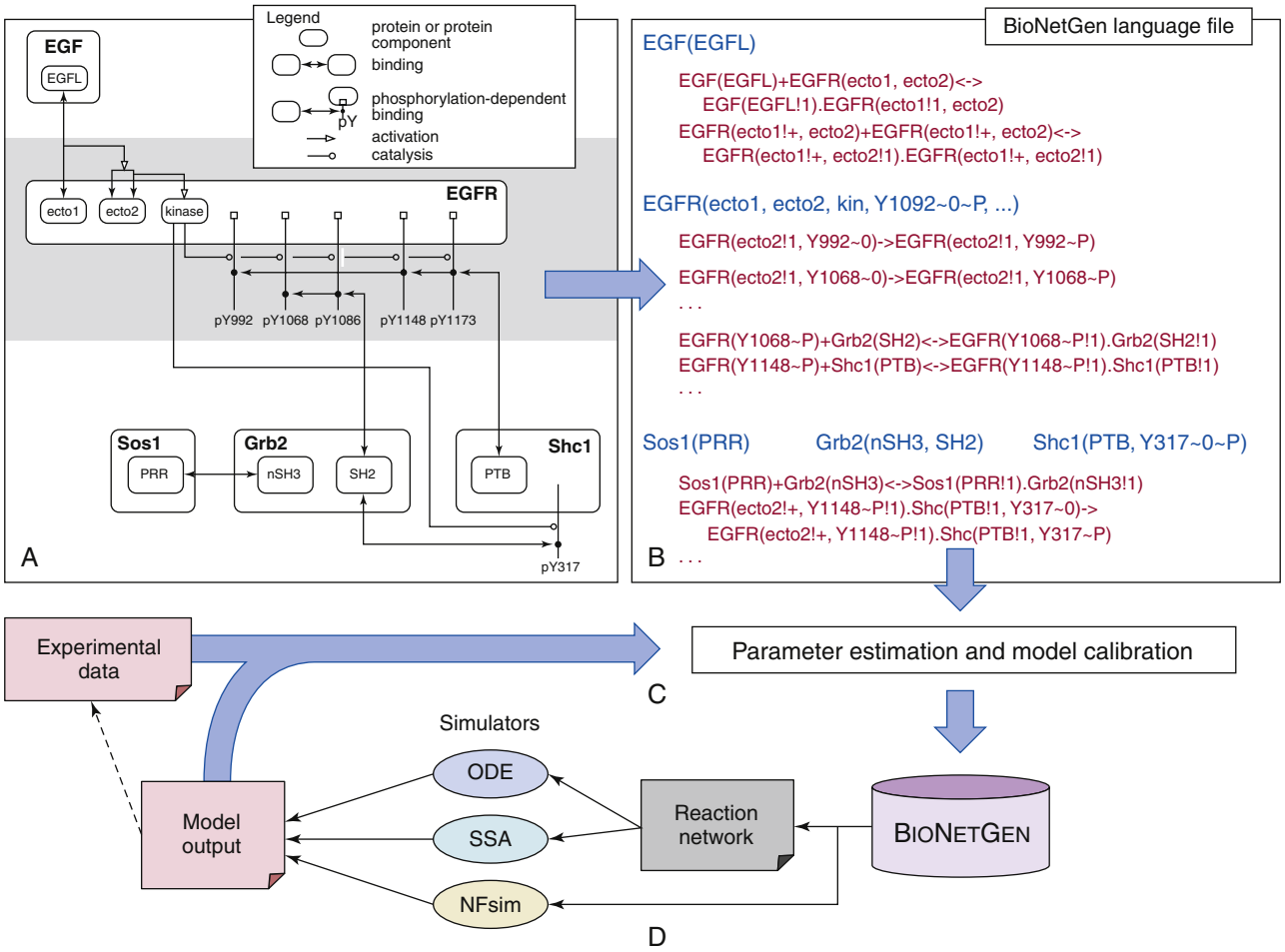


Figure 25-9 Steps in rule-based modeling of intracellular signaling Building a pathway model, such as the epidermal growth factor receptor (EGFR) pathway involves the following steps: **(A)** Create a map of pathway components and interactions; **(B)** translate elements of the map into molecules *(blue)* and rules *(red)* using, for example, the syntax of the BioNetGen[266] modeling language; **(C)** estimate the parameters, such as initial concentrations of proteins and rate constants, and calibrate model; and **(D)** run simulations using one of several methods such as ordinary differential equations (ODE), stochastic simulation algorithms (SSA), or network-free simulation (NFsim). Steps **C** and **D** are frequently iterated as model predictions are used to drive experimental studies, and the resulting data are used to refine and recalibrate the model.

procedure for constructing a rule-based model, using the EGFR pathway as an example. Rule-based modeling languages like BioNetGen[266] bridge the gap between the design of the model and the mathematical definition of its computation. Mechanistic modeling is distinct from machine learning approaches in that the underlying models are biophysical and biochemical in origin rather than purely descriptive of the data.[267,268] The two approaches share a common problem, which is the identification of model parameters that are consistent with a given set of data.[269] This step is necessary to generate predictions from the model and to estimate the confidence or uncertainty in those predictions,[270] but is currently a major bottleneck in the development of all forms of mechanistic models. This problem is particularly acute for large-scale models that have potentially tens to hundreds of unknown parameters.[271,272]

Here we identify four main challenges for developing computational models that integrate HCS data: network complexity, cell-to-cell variability, spatial complexity, and multicellularity, which we consider in the following paragraphs.

Network Complexity

Mechanistic models of cell signaling are usually based on standard chemical kinetics descriptions that arise from treating the cell as a well-stirred chemical reactor. Such reaction network models of cellular processes can be simulated by numerical integration of differential equations (if the cellular concentrations are large enough to be approximated as continuous) or through kinetic Monte Carlo simulations (most commonly, the Gillespie algorithm[273]), if the noise arising from small molecular concentrations is important. This approach is typically used to model systems ranging in size from a few species to as many as a few hundred, although parameter estimation becomes problematic for models of such complexity. More coarse-grained simulation approaches, such as Boolean network[274] or fuzzy-logic approaches,[235] which may reduce the problem of parameter estimation, have also been used as a basis for automated methods of learning model structure, i.e., the rules that govern interactions among the model components. Recently, statistical methods for model selection have also been applied to reaction network models to determine model architectures most compatible with experimental data.[275]

A major problem in cancer modeling is to predict the effect of observed mutations on the network response. Because mutations tend to effect specific molecular interactions, achieving this predictive capability drives the development of increasingly complex models. Unfortunately, the reaction network approach suffers from the problem of combinatorial complexity, which results in an explosion of network size—the numbers of species and reactions that must be considered in the model—as the complexity of molecules and number of interactions grows. This problem makes manual specification of reaction networks, the standard approach, time consuming and ultimately prohibitive. The cost of simulations also grows rapidly with network size and can become prohibitive even for relatively small systems.[276] The recent development of rule-based modeling[277,278] largely eliminates the effects of combinatorial complexity in the specification and simulation of models, although the general issue of calibrating the parameters of large-scale biological models remains open.

Cell-to-Cell Variability

In principle the effects of cell-to-cell variability in protein expression and other basic parameters such as volume or surface area can be modeled using reaction network models. For example, the distribution of responses to a fixed stimulation of a cell population could be modeled if the distributions of the key signaling components affecting the response were known. Recently, protein expression levels have been measured on a genome scale in single cells and shown to largely follow simple distributions characterized by only a few moments, e.g., mean, variance, and skewness.[279] If the necessary antibodies are available, HCA data can also be used to parameterize expression level distributions for a cell population, and these parameters can be fed into a model to predict the distribution of responses. An important additional issue, which has not often been addressed, is how correlations in protein expression affect variability. In one study of T-cell signaling, positive correlation between expression levels of activating and inhibitory components of a signaling pathway was shown to reduce the effect of cell-to-cell variations in those proteins.[280] A number of studies have shown that variability in protein expression can lead to dramatic variation in cellular responses, leading to bimodal distributions of activity or outcome.[280-282] Such effects typically arise from nonlinear effects, such as positive feedback, but can have other origins. Cell populations may use such variability as a form of bet-hedging to avoid making costly decisions, such as whether to grow or undergo apoptosis, in response to possibly spurious signals.[283] In the context of cancer, the effect of such variability complicates the development of effective treatments.

Spatial Complexity

Imaging provides a wealth of information about the spatial localization of molecules within cells. This localization is critical to many signaling processes. The movement of molecules between cell compartments is a major component of information flow and signal processing, e.g., endocytosis and transport of complexes through nuclear pore complexes. A range of modeling and computational approaches have been developed to describe such effects.[284] At the simplest level, compartments can be introduced to reaction network

models to prevent interactions between components in different compartments.[285] Models based on partial differential equations (VCell[286]) or Brownian dynamics (MCell[287]; Smoldyn[288]; GFRD[289]) handle spatial effects at a much higher level of detail. Depending on the level of resolution that is chosen, inclusion of spatial effects increases the simulation cost and the difficulty of fitting model parameters. Currently these costs preclude the possibility of model selection in most cases. At the same time, software for spatial simulation is advancing rapidly and can be expected to play a more important role as the amount of HCA data increases.

Multicellularity

The most daunting challenge for modeling HCA data comes in handling cell-cell interactions and coupling those to the molecular scale. Cell behaviors are coupled through the secretion and uptake of ligands, competition for resources, and direct cell-cell contacts. Modeling these effects requires multilevel models that couple intracellular regulatory networks to mechanisms for cell-cell communication. Cells are typically modeled as agents in such models. In order to develop multilevel models, standard methods for modeling cells as discrete agents in a population must be combined with reaction network modeling methods. Currently there are relatively few software tools available for the construction of such models,[290] and most of the models that have been developed use special-purpose code. Use of multilevel modeling has grown in the past few years and has been accompanied by the development of several new frameworks for developing multilevel models, such as ML-rules[291] and chaste.[292] The major issues going forward will be the computational expense of simulating such models and the accompanying issues of model parameterization and model selection.

Recently, the first truly comprehensive model of cellular processes and their regulation was developed for *Mycoplasma genitalium*, one of the simplest known organisms.[293] The model demonstrates that a wide range of cell processes can be modeled and integrated into a single computational model, but the effort required to construct and simulate the current model, which describes the behavior of only a single cell under controlled conditions, demonstrates the magnitude of the challenges that lie ahead.

Conclusion and Outlook

The integration of all imaging modalities with genomics and proteomics will have an important impact on our understanding of the molecular basis of cancer. In particular, HCA coupled to computational biology will yield the necessary statistical analyses to define and understand the impact of heterogeneity in both diagnostics and therapeutics. Although challenging, the development and application of systems biology tools should make it feasible to begin modeling the complexities of cancer.

Acknowledgments

This work was supported in part from research grants from the State of Pennsylvania Tobacco Funds (DLT), NIH R01 GM075205IH (RFM), NIH-NIGMS R01 GM086238 (TL, Ivet Bahar, PI), NIH-NCRR UL1RR024153-05 (TL and JRF, Steve Reiss, PI), and NIH-NCI 1R21CA164433-01A1 (CC, Liu, PI), and Pennsylvania Department of Health Cure Program Grant RFA#10-07-03 (RC-T). The authors thank Andy Stern for discussions on cancer biology and drug discovery, Adrian Lee and Steffi Oesterreich for discussions on cancer biomarkers, Ivet Bahar for discussions on computational biology, Jennifer Grandis for insights in head and neck cancer and Cal33 cells used in creating Figure 25-3, and Kumiko Isse and Anthony J. Demetris for supplying the images used for the analysis shown in Figure 25-7.

Key References

2. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
3. Koboldt DC, Fulton RS, McLellan MD, et al. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
4. Gatza ML, Kung HN, Blackwell KL, et al. Analysis of tumor environmental response and oncogenic pathway activation identifies distinct basal and luminal features in HER2-related breast tumor subtypes. *Breast Cancer Res*. 2011;13:R62.
5. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.
6. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
7. Critchley-Thorne RJ, Yu HX, Lee PP. Immune signatures associated with the cancer bearing state. In: Marincola FM, Wang E, eds. *Signatures of Rejection*. New York, NY: Springer; 2010.
8. Chouaib S, Kieda C, Benlalam H, et al. Endothelial cells as key determinants of the tumor microenvironment: interaction with tumor cells, extracellular matrix and immune killer cells. *Crit Rev Immunol*. 2010;30:529-545.
9. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res*. 2010;316:1324-1331.
10. Ostrand-Rosenberg S, Sinha P, Beury DW, et al. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol*. 2012;22:275-281.
11. Chen J, Yao Y, Gong C, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer Cell*. 2011;19:541-555.

12. Mantovani A, Savino B, Locati M, et al. The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev*. 2010;21:27-39.
16. Critchley-Thorne RJ, Miller SM, Taylor DL, et al. Applications of cellular systems biology in breast cancer patient stratification and diagnostics. *Comb Chem High Throughput Screen*. 2009;12:860-869.
17. Buchser WJ, Laskow TC, Pavlik PJ, et al. Cell-mediated autophagy promotes cancer cell survival. *Cancer Res*. 2012;72:2970-2979.
18. Albeck JG, Burke JM, Aldridge BB, et al. Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol Cell*. 2008;30:11-25.
19. Cohen AA, Geva-Zatorsky N, Eden E, et al. Dynamic proteomics of individual cancer cells in response to a drug. *Science*. 2008;322:1511-1516.
20. Abraham VC, Taylor DL, Haskins JR. High-content screening applied to large-scale cell biology. *Trends Biotechnol*. 2003;22:15-22.
21. Giuliano KA, Chen Y-T, Taylor DL. High-content screening with siRNA optimizes a cell biological approach to drug discovery: defining the role of p53 activation in the cellular response to anticancer drugs. *J Biomol Screen*. 2004;9:557-568.
22. Benjamin ER, Pruthi F, Olanrewaju S, et al. Pharmacological characterization of recombinant N-type calcium channel (Cav2.2) mediated calcium mobilization using FLIPR. *Biochem Pharmacol*. 2006;72:770-782.
23. Giuliano KA, Cheung WS, Curran DP, et al. Systems cell biology knowledge created from high content screening. *Assay Drug Dev Technol*. 2005;3:501-514.
24. Shibue T, Brooks MW, Inan MF, et al. The outgrowth of micrometastases is enabled by the formation of filopodium-like protrusions. *Cancer Discov*. 2012;2:706-721.
25. Shi J, Orth JD, Mitchison T. Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. *Cancer Res*. 2008;68:3269-3276.
26. Evensen L, Micklem DR, Link W, et al. A novel imaging-based high-throughput screening approach to anti-angiogenic drug discovery. *Cytometry A*. 2010;77:41-51.
27. Gupta PB, Fillmore CM, Jiang G, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell*. 2011;146:633-644.
28. Beliveau A, Mott JD, Lo A, et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. *Genes Dev*. 2010;24:2800-2811.
29. Yates C, Shepard CR, Papworth G, et al. Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. In: Vande Woude G, Klein G, eds. *Advances in Cancer Research: Applications of Viruses for Cancer Therapy*. Salt Lake City, Utah: Academic Press; 2007:225-246.
31. Zal T, Chodaczek G. Intravital imaging of anti-tumor immune response and the tumor microenvironment. *Semin Immunopathol*. 2010;32:305-317.
32. Provenzano PP, Eliceiri KW, Keely PJ. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin Exp Metastasis*. 2009;26:357-370.
33. Niesner RA, Hauser AE. Recent advances in dynamic intravital multi-photon microscopy. *Cytometry A*. 2011;79A:789-798.
34. Boissonnas A, Fetler L, Zeelenberg IS, et al. In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. *J Exp Med*. 2007;204:345-356.
35. Alexander S, Koehl GE, Hirschberg M, et al. Dynamic imaging of cancer growth and invasion: a modified skin-fold chamber model 11. *Histochem Cell Biol*. 2008;130:1147-1154.
36. Koehl G, Gaumann A, Geissler EK. Intravital microscopy of tumor angiogenesis and regression in the dorsal skin fold chamber: mechanistic insights and preclinical testing of therapeutic strategies. *Clin Exp Metastasis*. 2009;26:329-344.
37. Kedrin D, Wyckoff J, Sahai E, et al. Imaging tumor cell movement in vivo. *Curr Protoc Cell Biol*. 2007;19:7.
38. Kedrin D, Gligorijevic B, Wyckoff J, et al. Intravital imaging of metastatic behavior through a mammary imaging window. *Nat Methods*. 2008;5:1019-1021.
39. Lang P, Yeow K, Nichols A, et al. Cellular imaging in drug discovery. *Nat Rev Drug Discov*. 2006;5:343-356.
40. Hoelder S, Clarke PA, Workman P. Discovery of small molecule cancer drugs: successes, challenges and opportunities. *Mol Oncol*. 2012;6:155-176.
41. Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? *Br J Pharmacol*. 2010;160:204-216.
42. Pereira C, Coutinho I, Soares J, et al. New insights into cancer-related proteins provided by the yeast model. *FEBS J*. 2012;279:697-712.
43. Barberis A, Gunde T, Berset C, et al. Yeast as a screening tool. *Drug Discov Today*. 2005;2:187-192.
44. Ohnuki S, Oka S, Nogami S, et al. High-content, image-based screening for drug targets in yeast. *PLoS One*. 2010;5:e10177.
45. Vizeacoumar F, van Dyk N, Vizeacoumar FS, et al. Integrating high-throughput genetic interaction mapping and high-content screening to explore yeast spindle morphogenesis. *J Cell Biol*. 2010;188:69-81.
46. Siddiqui SS, Loganathan S, Krishnaswamy S, et al. *C. elegans* as a model organism for in vivo screening in cancer: effects of human c-Met in lung cancer affect *C. elegans* vulva phenotypes. *Cancer Biol Ther*. 2008;7:856-863.
47. Bae Y-K, Sung JY, Kim Y-N, et al. An in vivo *C. elegans* model system for screening EGFR-inhibiting anti-cancer drugs. *PLoS One*. 2012;7:1-9.
48. Matus DQ, Li X-Y, Durbin S, et al. In vivo identification of regulators of cell invasion across basement membranes. *Sci Signal*. 2010;3:1-9.
49. Rudrapatna VA, Cagan RL, Das TK. Drosophila cancer models. *Dev Dynam*. 2012;241:1-12.
50. Miles WO, Dyson NJ, Walker JA. Modeling tumor invasion and metastasis in Drosophila. *Dis Models Mech*. 2011;4:753-761.
51. Chen C-L, Schroeder MC, Kango-Singh M, et al. Tumor suppression by cell competition through regulation of the Hippo pathway. *Proc Natl Acad Sci U S A*. 2012;109:484-489.
52. Dar AC, Das TK, Shokat KM, et al. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature*. 2012;486:80-85.
53. Binder V, Zon LI. High throughput in vivo phenotyping: the zebrafish as a tool for drug discovery for hematopoietic stem cells and cancer. 10.1016/j.ddmod.2012.02.007.
54. Feitsma H, Cuppen E. Zebrafish as a cancer model. *Mol Cancer Res*. 2008;6:685-694.
55. Taylor AM, Zon LI. Zebrafish tumor assays: the state of transplantation. *Zebrafish*. 2009;6:339-346.
56. Vogt A, Cholewinski A, Shen X, et al. Automated image-based phenotypic analysis in zebrafish embryos. *Dev Dynam*. 2009;238:656-663.
57. Prescher JA, Contag CH. Guided by the light: visualizing biomolecular processes in living animals with bioluminescence. *Curr Opin Chem Biol*. 2010;14:80-89.
58. Pittet MJ, Weissleder R. Intravital imaging. *Cell*. 2011;147:983-991.
59. Croix CS, Zipfel WR, Watkins SC. Potential solutions for confocal imaging of living animals. *Biotechniques*. 2007;43:14-19.
60. Kaijzel EL, van der Pluijm G, Löwen Clemens WGM. Whole-body optical imaging in animal models to assess cancer development and progression. *Clin Cancer Res*. 2007;13:3490-3497.
62. Giuliano KA, Haskins JR, Taylor DL. Advances in high content screening for drug discovery. *Assays Drug Dev Tech*. 2003;1:565-577.
63. Giuliano KA, Taylor DL, Waggoner AS. Reagents to measure and manipulate cell functions. In: *High Content Screening*. Totowa, NJ: Humana Press; 2006:141-163.
64. Taylor DL. Past, present, and future of high content screening and the field of cellomics. *Methods Mol Biol*. 2007;356:3-18.
65. Haney SA, ed. *High Content Screening: Science, Techniques, and Applications*. New York, NY: Wiley; 2008.
66. Inglese J, ed. *Methods in Enzymology: Vol 414. Measuring Biological Responses with Automated Microscopy*. New York, NY: Academic Press; 2006.
67. Taylor D, Haskins J, Giuliano K, eds. *Methods in Molecular Biology: Vol 356. High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery*. New York, NY: Springer; 2007.
68. Bullen A. Microscopic imaging techniques for drug discovery. *Nat Rev Drug Discov*. 2008;7:54-67.
69. Thomas N. High-content screening: a decade of evolution. *J Biomol Screen*. 2010;15:1-7.
70. Critchley-Thorne RJ, Miller SM, Taylor DL, et al. Applications of cellular systems biology in breast cancer patient stratification and diagnostics. *Comb Chem High Throughput Screen*. 2009;12:860-869.
71. Zanella F, Lorens JB, Link W. High content screening: seeing is believing. *Trends Biotechnol*. 2010;28:237-245.

72. Zock J. Applications of high content screening in life science research. *Comb Chem High Throughput Screen*. 2009;12:870-876.
73. Purdue University Cytometry Laboratory. *Antibodies, Reagents, Fluorescent Probes*. Retrieved from http://www.cyto.purdue.edu/flowcyt/supplier/reagents.htm.
74. Salk Institute for Biological Studies. *Table of Fluorochromes*. Retrieved from http://flowcyt.salk.edu/fluo.html.
75. Spence MTZ, Johnson ID. *The Molecular Probes Handbook: a Guide to Fluorescent Probes and Labeling Technologies*. 11th ed. Carlsbad, CA: Life Technologies Corp; 2010.
76. Fleuret F, Li T, Dubout C, et al. Comparing machines and humans on a visual categorization test. *Proc Natl Acad Sci U S A*. 2011;108:17621-17625.
77. Wood KC, Konieczkowski DJ, Johannessen CM, et al. MicroSCALE screening reveals genetic modifiers of therapeutic response in melanoma. *Sci Signal*. 2012;5:1-12.
78. Cheong R, Paliwal S, Levchenko A. High content screening in microfluidic devices. *Expert Opin Drug Discov*. 2010;5:715-720.
79. Fuchs F, Pau G, Kranz D, et al. Clustering phenotype populations by genome-wide RNAi and multiparametric imaging. *Mol Syst Biol*. 2010;6:370.
80. Murphy RF. An active role for machine learning in drug development. *Nat Chem Biol*. 2011;7:327-330.
81. Sasaki K, Ito T, Nishino N. Real-time imaging of histone H4 hyperacetylation in living cells. *Proc Natl Acad Sci U S A*. 2009;106:16257-16262.
82. Ashall L, Horton CA, Nelson DE. Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science*. 2009;324:242-246.
83. Kalén M, Wallgard E, Asker N. Combination of reverse and chemical genetic screens reveals angiogenesis inhibitors and targets. *Chem Biol*. 2009;16:432-441.
84. Livesey KM, Kang R, Vernon P, et al. p53/HMGB1 complexes regulate autophagy and apoptosis. *Cancer Res*. 2012;72:1996-2005.
85. Norton J, Titus SA, Dexter D. Automated high content screening for compounds that disassemble the perinucleolar compartment. *J Biomol Screen*. 2009;14:1045-1053.
86. Raccor B, Vogt A, Sikorski RP. Cell-based and biochemical structure-activity analyses of analogs of the microtubule stabilizer dictyostatin. *Mol Pharmacol*. 2008;73:718-726.
87. Conrad C, Gerlich DW. Automated microscopy for high-content RNAi screening. *J Cell Biol*. 2010;188:453-461.
88. Moffat J, Sabatini DM. Building mammalian signalling pathways with RNAi screens. *Nat Rev Mol Cell Biol*. 2006;7:177-187.
89. Rines D, Gomez-Ferreria MA, Zhou Y. Whole genome functional analysis identifies novel components required for mitotic spindle integrity in human cells. *Genome Biol*. 2008;9:R44.
90. Antczak C, Takagi T, Ramirez CN, et al. Live-cell imaging of caspase activation for high-content screening. *J Biomol Screen*. 2009;14:956-969.
91. Guzi T, Paruch K, Dwyer MP, et al. Targeting the replication checkpoint using SCH 900776, a potent and functionally selective CHK1 inhibitor identified via high content screening. *Mol Cancer Ther*. 2011;10:591-602.
92. Neumann B, Walter T, Hériché JK. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature*. 2010;464:721-727.
93. Schmitz M, Held M, Janssens V, et al. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells 8. *Nat Cell Biol*. 2010;12:886-893.
95. Haney S, LaPan P, Pan J, et al. High content screening moves to the front of the line. *Drug Discov Today*. 2006;11:889-894.
96. Bickle M. The beautiful cell: high-content screening in drug discovery 2. *Anal Bioanal Chem*. 2010;398:219-226.
97. Birsoy K, Sabatini DM, Possemato R. Targeting cancer metabolism: a bedside lesson. *Nat Med*. 2012;18:1022-1023.
98. Arora R, Shuda M, Gustafierro A, et al. Viral knockdown reveals surviving as a therapeutic target for Merkel cell carcinoma. *Science Transl Med*. 2012;4:133ra56.
99. Xu G, Mawji I, Macrae C, et al. A high-content chemical screen identifies ellipticine as a modulator of p53 nuclear localization. *Apoptosis*. 2008;13:413-422.
100. Molina G, Vogt A, Bakan A, et al. Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. *Nat Chem Biol*. 2009;5:680-687.
101. Danli L, Nicholl EE, Comess KM, et al. Development of a high-content screening assay panel to accelerate mechanism of action studies for oncology research. *J Biomol Screen*. 2012;17:1005-1017.
102. Anagnostou VK, Dimou AT, Botsis T, et al. Molecular classification of nonsmall cell lung cancer using a 4-protein quantitative assay. *Cancer*. 2012;118:1607-1618.
103. Link W, Oyarzabal J, Serelde BG. Chemical interrogation of FOXO3a nuclear translocation identifies potent and selective inhibitors of phosphoinositide 3-kinases. *J Biol Chem*. 2009;284:28392-28400.
104. Zanella F, Rosado A, García B. Chemical genetic analysis of FOXO nuclear-cytoplasmic shuttling by using image-based cell screening. *Chembiochem*. 2008;9:2229-2237.
105. Loo L-H, Wu LF, Altschuler SJ. Image-based multivariate profiling of drug responses from single cells. *Nat Methods*. 2007;4:445-453.
106. Perlman ZE, Slack MD, Feng Y, et al. Multidimensional drug profiling by automated microscopy. *Science*. 2004;306:1194-1198.
107. Schreiber S, Kapoor TM, Wess G. *Chemical Biology: From Small Molecules to Systems Biology and Drug Design*. Weinheim, Germany: Wiley-VCH; 2007.
109. Sachs K, Perez O, Pe'er D, et al. Causal protein-signaling networks derived from multiparameter single-cell data. *Science*. 2005;308:523-529.
110. Xing Y, Chaudry Q, Shen C, et al. Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. *Nat Protoc*. 2007;2:1152-1165.
111. Barash E, Dinn S, Sevinsky C, et al. Multiplexed analysis of proteins in tissue using multispectral fluorescence imaging. *IEEE Trans Med Imaging*. 2010;29:1457-1462.
112. Mittag A, Lenz D, Bocsi J, et al. Sequential photobleaching of fluorochromes for polychromatic slide-based cytometry. *Cytometry A*. 2006;69:139-141.
114. Schubert W, Bonnekoh B, Pommer AJ, et al. Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat Biotechnol*. 2006;24:1270-1278.
115. Schubert W, Gieseler A, Krusche A, et al. Next-generation biomarkers based on 100-parameter functional super-resolution microscopy TIS. *N Biotechnol*. 2012;29:599-610.
116. Gerdes MJ, Sevinsky CJ, Sood A, et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc Natl Acad Sci U S A*. 2013;110:11982-11987.
117. Esposito A, Wouters FS. Fluorescence lifetime imaging microscopy. *Curr Protoc Cell Biol*. 2004 4.14.
118. Setou M. Imaging Mass Spectrometry: Protocols for Mass Spectrometry. New York: Springer, 2010
119. Riemann B, Schafers KP, Schober O, et al. Small animal PET in preclinical studies: opportunities and challenges. *Q J Nucl Med Mol Imaging*. 2008;52:215-221.
120. Vande Velde G, Baekelandt V, Dresselaers T. Magnetic resonance imaging and spectroscopy methods for molecular imaging. *Q J Nucl Med Mol Imaging*. 2009;53:565-585.
121. Kraljevic S, Sedic M, Scott M, et al. Casting light on molecular events underlying anti-cancer drug treatment: what can be seen from the proteomics point of view? *Cancer Treat Rev*. 2006;32:619-629.
122. Turner NC, Reis-Filho JS. Genetic heterogeneity and cancer drug resistance. *Lancet Oncol*. 2012;13:e178-e185.
123. Niepel M, Spencer SL, Sorger PK. Non-genetic cell-to-cell variability and the consequences for pharmacology. *Curr Opin Chem Biol*. 2009;13:556-561.
124. Spencer SL, Gaudet S, Albeck JG, et al. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature*. 2009;459:428-432.
125. Huang S. Non-genetic heterogeneity of cells in development: more than just noise. *Development*. 2009;136:3853-3862.
127. Wang D, Bodovitz S. Single cell analysis: the new frontier in "omics". *Trends Biotechnol*. 2010;28:281-290.
128. Towne DL, Nicholl EE, Comess KM, et al. Development of a high-content screening assay panel to accelerate mechanism of action studies for oncology research. *J Biomol Screen*. 2012;17:1005-1017.
129. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat*. 2004;83:249-289.
130. Clark MJ, Homer N, O'Connor BD, et al. U87MG decoded: the genomic sequence of a cytogenetically aberrant human cancer cell line. *PLoS Genet*. 2010;6:e1000832.
131. Borrell B. How accurate are cancer cell lines? *Nature*. 2010;463:858.

132. Gillet J-P, Calcagno AM, Varma S, et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci U S A*. 2011;108:18708-18713.
133. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-307.
134. Neve RM, Chin K, Fridlyand J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. 2006;10:515-527.
135. Weinstein JN. Drug discovery: cell lines battle cancer. *Nature*. 2012;483:544-545.
136. Reinhold WC, Sunshine M, Liu H, et al. CellMiner: a web-based suite of genomic and pharmacologic tools to explore transcript and drug patterns in the NCI-60 cell line set. *Cancer Res*. 2012;72:3499-3511.
137. Liu H, D'Andrade P, Fulmer-Smentek S, et al. mRNA and microRNA expression profiles of the NCI-60 integrated with drug activities. *Mol Cancer Ther*. 2010;9:1080-1091.
138. National Cancer Institute. *The Cancer Genome Atlas*. Retrieved from http://cancergenome.nih.gov/publications.
139. Pandita A, Aldape KD, Zadeh G, et al. Contrasting in vivo and in vitro fates of glioblastoma cell subpopulations with amplified EGFR. *Genes Chromosomes Cancer*. 2004;39:29-36.
140. Keller PJ, Lin AF, Arendt LM, et al. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. *Breast Cancer Res*. 2010;12:R87.
142. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009;4:e6146.
143. Giuliano KA, Johnston PA, Gough A, et al. Systems cell biology based on high-content screening. *Methods Enzymol*. 2006;414:601-619.
144. Kim H, Phung Y, Ho M. Changes in global gene expression associated with 3D structure of tumors: an ex vivo matrix-free mesothelioma spheroid model. *PLoS One*. 2012;7:e39556.
145. Kunz-Schughart LA, Freyer JP, Hofstaedter F, et al. The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *J Biomol Screen*. 2004;9:273-285.
146. Kenny PA, Lee GY, Myers CA, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol*. 2007;1:84-96.
147. Gautrot JE, Wang C, Liu X, et al. Mimicking normal tissue architecture and perturbation in cancer with engineered micro-epidermis. *Biomaterials*. 2012;33:5221-5229.
148. Pinto MP, Jacobsen BM, Horwitz KB. An immunohistochemical method to study breast cancer cell subpopulations and their growth regulation by hormones in three-dimensional cultures. *Front Endocrinol (Lausanne)*. 2011;2:15.
149. Leung CT, Brugge JS. Outgrowth of single oncogene-expressing cells from suppressive epithelial environments. *Nature*. 2012;482:410-413.
150. Norton KA, Namazi S, Barnard N, et al. Automated reconstruction algorithm for identification of 3D architectures of cribriform ductal carcinoma in situ. *PLoS One*. 2012;7:e44011.
151. Caicedo-Carvajal CE, Liu Q, Remache Y, et al. Cancer tissue engineering: a novel 3D polystyrene scaffold for in vitro isolation and amplification of lymphoma cancer cells from heterogeneous cell mixtures. *J Tissue Eng*. 2011;2011:362326.
152. Dhiman HK, Ray AR, Panda AK. Three-dimensional chitosan scaffold-based MCF-7 cell culture for the determination of the cytotoxicity of tamoxifen. *Biomaterials*. 2005;26:979-986.
153. Dutta RC, Dutta AK. Cell-interactive 3D-scaffold; advances and applications. *Biotechnol Adv*. 2009;27:334-339.
154. Dutta RC. In search of optimal scaffold for regenerative medicine and therapeutic delivery. *Ther Deliv*. 2011;2:231-234.
155. Timpson P, McGhee EJ, Anderson KI. Imaging molecular dynamics in vivo—from cell biology to animal models. *J Cell Sci*. 2011;124:2877-2890.
156. Gil V, del Rio JA. Analysis of axonal growth and cell migration in 3D hydrogel cultures of embryonic mouse CNS tissue. *Nat Protoc*. 2012;7:268-280.
157. Ziolkowska K, Kwapiszewski R, Brzozka Z. Microfluidic devices as tools for mimicking the in vivo environment. *N J Chem*. 2011;35:979-990.
158. Vinci M, Gowan S, Boxall F, et al. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol*. 2012;10:29.
159. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res*. 2011;13:215.
160. Altschuler SJ, Wu LF. Cellular heterogeneity: do differences make a difference? *Cell*. 2010;141:559-563.
161. Merlo LMF, Pepper JW, Reid BJ, et al. Cancer as an evolutionary and ecological process. *Nat Rev Cancer*. 2006;6:924-935.
162. Dalerba P, Kalisky T, Sahoo D, et al. Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat Biotechnol*. 2011;29:1120-1127.
163. Houston MA, Augenlicht LH, Heerdt BG. Stable differences in intrinsic mitochondrial membrane potential of tumor cell subpopulations reflect phenotypic heterogeneity. *Int J Cell Biol*. 2011;2011:978583.
164. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
165. Singh DK, Ku CJ, Wichaidit C, et al. Patterns of basal signaling heterogeneity can distinguish cellular populations with different drug sensitivities. *Mol Syst Biol*. 2010;6:369.
166. Feng Y, Mitchison TJ, Bender A, et al. Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds. *Nat Rev Drug Discov*. 2009;8:567-578.
167. Giuliano KA, Gough AH, Taylor DL, et al. Early safety assessment using cellular systems biology yields insights into mechanisms of action. *J Biomol Screen*. 2010;15:783-797.
168. Kummel A, Selzer P, Siebert D, et al. Differentiation and visualization of diverse cellular phenotypic responses in primary high-content screening. *J Biomol Screen*. 2012;17:843-849.
169. Jones TR, Carpenter AE, Lamprecht MR, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A*. 2009;106:1826-1831.
170. Ng AY, Rajapakse JC, Welsch RE, et al. A cell profiling framework for modeling drug responses from HCS imaging. *J Biomol Screen*. 2010;15:858-868.
171. Loo LH, Lin HJ, Steininger 3rd RJ, et al. An approach for extensibly profiling the molecular states of cellular subpopulations. *Nat Methods*. 2009;6:759-765.
174. Souissi I, Ladam P, Cognet JA, et al. A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line. *Mol Cancer*. 2012;11:12.
175. Rexhepaj E, Brennan DJ, Holloway P, et al. Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: application to measurement of oestrogen and progesterone receptor levels in breast cancer. *Breast Cancer Res*. 2008;10:R89.
176. Sharangpani GM, Joshi AS, Porter K, et al. Semi-automated imaging system to quantitate estrogen and progesterone receptor immunoreactivity in human breast cancer. *J Microsc*. 2007;226:244-255.
177. Huang W, Hennrick K, Drew S. A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Hum Pathol*. 2013;44:29-38.
178. Chakravarti A, DeSilvio M, Zhang M, et al. Prognostic value of p16 in locally advanced prostate cancer: a study based on Radiation Therapy Oncology Group Protocol 9202. *J Clin Oncol*. 2007;25:3082-3089.
179. Nassar A, Cohen C, Agersborg SS, et al. A multisite performance study comparing the reading of immunohistochemical slides on a computer monitor with conventional manual microscopy for estrogen and progesterone receptor analysis. *Am J Clin Pathol*. 2011;135:461-467.
180. Nassar A, Cohen C, Agersborg SS, et al. A new immunohistochemical ER/PR image analysis system: a multisite performance study. *Appl Immunohistochem Mol Morphol*. 2011;19:195-202.
181. Jeung J, Patel R, Vila L, et al. Quantitation of HER2/neu expression in primary gastroesophageal adenocarcinomas using conventional light microscopy and quantitative image analysis. *Arch Pathol Lab Med*. 2012;136:610-617.
182. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology*. 2006;49:411-424.
183. Leong AS. Pitfalls in diagnostic immunohistology. *Adv Anat Pathol*. 2004;11:86-93.

184. Jain RK, Mehta R, Dimitrov R, et al. Atypical ductal hyperplasia: interobserver and intraobserver variability. *Mod Pathol*. 2011;24:917-923.
185. Castro NP, Osorio CA, Torres C, et al. Evidence that molecular changes in cells occur before morphological alterations during the progression of breast ductal carcinoma. *Breast Cancer Res*. 2008;10:R87.
186. Nederlof M, Watanabe S, Burnip B, et al. High-throughput profiling of tissue and tissue model microarrays: combined transmitted light and 3-color fluorescence digital pathology. *J Pathol Inform*. 2011;2:50.
187. Laurinavicius A, Laurinaviciene A, Ostapenko V, et al. Immunohistochemistry profiles of breast ductal carcinoma: factor analysis of digital image analysis data. *Diagn Pathol*. 2012;7:27.
188. Tadrous PJ. On the concept of objectivity in digital image analysis in pathology. *Pathology*. 2010;42:207-211.
189. Madabhushi A. Digital pathology image analysis: opportunities and challenges. *Imaging Med*. 2009;1:7-10.
190. Robertson D, Savage K, Reis-Filho JS, et al. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biol*. 2008;9:13.
191. Donovan MJ, Khan FM, Bayer-Zubek V, et al. A systems-based modelling approach using transurethral resection of the prostate (TURP) specimens yielded incremental prognostic significance to Gleason when predicting long-term outcome in men with localized prostate cancer. *BJU Int*. 2012;109:207-213.
192. Wang J, Barnes RO, West NR, et al. Jab1 is a target of EGFR signaling in ERalpha-negative breast cancer. *Breast Cancer Res*. 2008;10:R51.
194. Giltnane JM, Molinaro A, Cheng H, et al. Comparison of quantitative immunofluorescence with conventional methods for HER2/neu testing with respect to response to trastuzumab therapy in metastatic breast cancer. *Arch Pathol Lab Med*. 2008;132:1635-1647.
195. Bloom K, Harrington D. Enhanced accuracy and reliability of HER-2/neu immunohistochemical scoring using digital microscopy. *Am J Clin Pathol*. 2004;121:620-630.
196. Gustavson MD, Bourke-Martin B, Reilly D, et al. Standardization of HER2 immunohistochemistry in breast cancer by automated quantitative analysis. *Arch Pathol Lab Med*. 2009;133:1413-1419.
197. Sempere LF, Preis M, Yezefski T, et al. Fluorescence-based codetection with protein markers reveals distinct cellular compartments for altered microRNA expression in solid tumors. *Clin Cancer Res*. 2010;16:4246-4255.
198. Shachaf CM, Elchuri SV, Koh AL, et al. A novel method for detection of phosphorylation in single cells by surface enhanced Raman scattering (SERS) using composite organic-inorganic nanoparticles (COINs). *PLoS One*. 2009;4:e5206.
199. Bhattacharya S, Mathew G, Ruban E, et al. Toponome imaging system: in situ protein network mapping in normal and cancerous colon from the same patient reveals more than five-thousand cancer specific protein clusters and their subcellular annotation by using a three symbol code. *J Proteome Res*. 2012;9:6112-6125.
200. Fernandez DC, Bhargava R, Hewitt SM, et al. Infrared spectroscopic imaging for histopathologic recognition. *Nat Biotechnol*. 2005;23:469-474.
201. Freudiger CW, Min W, Saar BG, et al. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. *Science*. 2008;322:1857-1861.
202. Yang Y, Li F, Gao L, et al. Differential diagnosis of breast cancer using quantitative, label-free and molecular vibrational imaging. *Biomed Opt Express*. 2011;2:2160-2174.
203. Bird B, Miljkovic MS, Remiszewski S, et al. Infrared spectral histopathology (SHP): a novel diagnostic tool for the accurate classification of lung cancer. *Lab Invest*. 2012;92:1358-1373.
204. Kwak JT, Hewitt SM, Sinha S, et al. Multimodal microscopy for automated histologic analysis of prostate cancer. *BMC Cancer*. 2011;11:62.
205. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol*. 2007;25:5287-5312.
206. McCabe A, Dolled-Filhart M, Camp RL, et al. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. *J Natl Cancer Inst*. 2005;97:1808-1815.
207. Cuzick J, Dowsett M, Pineda S, et al. Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. *J Clin Oncol*. 2011;29:4273-4278.
208. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med*. 2004;351:2817-2826.
209. Paik S. Development and clinical utility of a 21-gene recurrence score prognostic assay in patients with early breast cancer treated with tamoxifen. *Oncologist*. 2007;12:631-635.
210. Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). *Clin Breast Cancer*. 2006;7:347-350.
212. Mook S, Van't Veer LJ, Rutgers EJ, et al. Individualization of therapy using Mammaprint: from development to the MINDACT Trial. *Cancer Genomics Proteomics*. 2007;4:147-155.
213. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008;14:518-527.
214. Van Laere SJ, Van der Auwera I, Van den Eynden GG, et al. NF-kappaB activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. *Br J Cancer*. 2007;97:659-669.
215. Dolled-Filhart M, Camp RL, Kowalski DP, et al. Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Clin Cancer Res*. 2003;9:594-600.
216. Mefford D, Mefford J. Stromal genes add prognostic information to proliferation and histoclinical markers: a basis for the next generation of breast cancer gene signatures. *PLoS One*. 2012;7:e37646.
217. Robinson BD, Sica GL, Liu YF, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res*. 2009;15:2433-2441.
218. Ladanyi A, Kiss J, Somlai B, et al. Density of DC-LAMP$^+$ mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. *Cancer Immunol Immunother*. 2007;56:1459-1469.
219. Oktay MH, Gertler FB, Liu YF, et al. Correlated immunohistochemical and cytological assays for the prediction of hematogenous dissemination of breast cancer. *J Histochem Cytochem*. 2012;60:168-173.
220. Medrek C, Ponten F, Jirstrom K, et al. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer*. 2012;12:306.
222. Honeth G, Bendahl PO, Ringner M, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res*. 2008;10:R53.
223. Neumeister V, Agarwal S, Bordeaux J, et al. In situ identification of putative cancer stem cells by multiplexing ALDH1, CD44, and cytokeratin identifies breast cancer patients with poor prognosis. *Am J Pathol*. 2010;176:2131-2138.
224. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24:5373-5380.
225. Weise A, Timmermann B, Grabherr M, et al. High-throughput sequencing of microdissected chromosomal regions. *Eur J Hum Genet*. 2010;18:457-462.
226. Cha S, Imielinski MB, Rejtar T, et al. In situ proteomic analysis of human breast cancer epithelial cells using laser capture microdissection: annotation by protein set enrichment analysis and gene ontology. *Mol Cell Proteomics*. 2010;9:2529-2544.
227. Finkelstein SD, Marsh W, Demetris AJ, et al. Microdissection-based allelotyping discriminates de novo tumor from intrahepatic spread in hepatocellular carcinoma. *Hepatology*. 2003;37:871-879.
228. Johann DJ, Rodriguez-Canales J, Mukherjee S, et al. Approaching solid tumor heterogeneity on a cellular basis by tissue proteomics using laser capture microdissection and biological mass spectrometry. *J Proteome Res*. 2009;8:2310-2318.
230. Yang F, Foekens JA, Yu J, et al. Laser microdissection and microarray analysis of breast tumors reveal ER-alpha related genes and pathways. *Oncogene*. 2006;25:1413-1419.
231. Wittliff JL, Kruer TL, Andres SA, et al. Molecular signatures of estrogen receptor-associated genes in breast cancer predict clinical outcome. *Adv Exp Med Biol*. 2008;617:349-357.
232. Gregg JL, Brown KE, Mintz EM, et al. Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection. *BMC Cancer*. 2010;10:165.
233. Dietrich D, Lesche R, Tetzner R, et al. Analysis of DNA methylation of multiple genes in microdissected cells from formalin-fixed and paraffin-embedded tissues. *J Histochem Cytochem*. 2009;57:477-489.

234. Gagnon JF, Sanschagrin F, Jacob S, et al. Quantitative DNA methylation analysis of laser capture microdissected formalin-fixed and paraffin-embedded tissues. *Exp Mol Pathol*. 2010;88:184-189.
235. Morris MK, Saez-Rodriguez J, Clarke DC, et al. Training signaling pathway maps to biochemical data with constrained fuzzy logic: quantitative analysis of liver cell responses to inflammatory stimuli. *PLoS Comput Biol*. 2011;7:e1001099.
237. Nandy K, Gudla PR, Amundsen R, et al. Automatic segmentation and supervised learning-based selection of nuclei in cancer tissue images. *Cytometry A*. 2012;81:743-754.
238. Ginty F, Adak S, Can A, et al. The relative distribution of membranous and cytoplasmic met is a prognostic indicator in stage I and II colon cancer. *Clin Cancer Res*. 2008;14:3814-3822.
239. Baatz M, Arini N, Schape A, et al. Object-oriented image analysis for high content screening: detailed quantification of cells and sub cellular structures with the Cellenger software. *Cytometry A*. 2006;69:652-658.
240. Tredan O, Galmarini CM, Patel K, et al. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst*. 2007;99:1441-1454.
241. Chennubhotla C, Jepson A. EigenCuts: Half-lives of eigenflows for spectral clustering. *NIPS*. 2003;15:689-896.
242. Chennubhotla C, Jepson A. Hierarchical eigensolver for transition matrices in spectral methods. *NIPS*. 2005;17:273-280.
243. Burger V, Chennubhotla CNHS. network-based hierarchical segmentation for cryo-electron microscopy density maps. *Biopolymers*. 2012;97:732-741.
244. Mitchell T. Machine learning and data mining. *Commun ACM*. 1999;42:31-36.
249. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med*. 2003;9:416-423.
250. Meinel LA, Stolpen AH, Berbaum KS, et al. Breast MRI lesion classification: improved performance of human readers with a backpropagation neural network computer-aided diagnosis (CAD) system. *J Magn Reson Imaging*. 2007;25:89-95.
251. Beck AH, Sangoi AR, Leung S, et al. Systematic analysis of breast cancer morphology uncovers stromal features associated with survival. *Sci Transl Med*. 2011;3:108ra113.
253. Glory E, Newberg J, Murphy RF. Automated comparison of protein subcellular location patterns between images of normal and cancerous tissues. *Proc IEEE Int Symp Biomed Imaging*. 2008;4540993:304-307.
254. Ellison DW, Onilude OE, Lindsey JC, et al. beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. *J Clin Oncol*. 2005;23:7951-7957.
257. Chen X, Velliste M, Weinstein S, et al. Location proteomics—building subcellular location trees from high resolution 3D fluorescence microscope images of randomly-tagged proteins. *Proc SPIE*. 2003;4962:298-306.
258. Slack MD, Martinez ED, Wu LF, et al. Characterizing heterogeneous cellular responses to perturbations. *Proc Natl Acad Sci U S A*. 2008;105:19306-19311.
259. Giuliano KA, Premkumar D, Strock CJ, et al. Cellular systems biology profiling applied to cellular models of disease. *Comb Chem High Throughput Screen*. 2009;12:838-848.
264. Chennubhotla C, Jepson A. Half-lives of eigenflows for spectral clustering. In: Becker S, Thrun S, Obermayer K, eds. *Advances in Neural Information Processing Systems*. Cambridge, MA: MIT Press; 2003:689-696.
265. Chennubhotla C, Jepson A. Hierarchical eigensolver for transition matrices in spectral methods. In: Saul L, Weiss Y, Bottou L, eds. *Advances in Neural Information Processing Systems*. Cambridge, MA: MIT Press; 2005:273-280.
266. Faeder JR, Blinov ML, Hlavacek WS. Rule-based modeling of biochemical systems with BioNetGen. In: Maly IV, ed. *Methods in Molecular Biology: Systems Biology*. Totowa, NJ: Humana Press; 2009.
267. Aldridge BB, Burke JM, Lauffenburger DA, et al. Physicochemical modelling of cell signalling pathways. *Nat Cell Biol*. 2006;8:1195-1203.
268. Kholodenko BN. Cell-signalling dynamics in time and space. *Nat Rev Mol Cell Biol*. 2006;7:165-176.
269. Chen WW, Niepel M, Sorger PK. Classic and contemporary approaches to modeling biochemical reactions. *Genes Dev*. 2010;24:1861-1875.
270. Jaqaman K, Danuser G. Linking data to models: data regression. *Nat Rev Mol Cell Biol*. 2006;7:813-819.
271. Chen WW, Schoeberl B, Jasper PJ, et al. Input-output behavior of ErbB signaling pathways as revealed by a mass action model trained against dynamic data. *Mol Syst Biol*. 2009;5:239.
272. Hlavacek WS. How to deal with large models? *Mol Syst Biol*. 2009;5:240.
273. Gillespie DT. Stochastic simulation of chemical kinetics. *Annu Rev Phys Chem*. 2007;58:35-55.
274. Wang RS, Saadatpour A, Albert R. Boolean modeling in systems biology: an overview of methodology and applications. *Phys Biol*. 2012;9:055001.
275. Gomez-Cabrero D, Compte A, Tegner J. Workflow for generating competing hypothesis from models with parameter uncertainty. *Interface Focus*. 2011;1:438-449.
276. Sneddon MW, Faeder JR, Emonet T. Efficient modeling, simulation and coarse-graining of biological complexity with NFsim. *Nat Methods*. 2011;8:177-183.
277. Hlavacek WS, Faeder JR, Blinov ML, et al. Rules for modeling signal-transduction systems. *Sci STKE*. 2006:re6.
278. Danos V, Feret J, Fontana W, et al. Rule-based modelling of cellular signalling. *Lect Notes Comput Sci*. 2007;4703:17-41.
279. Taniguchi Y, Choi PJ, Li GW, et al. Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science*. 2010;329:533-538.
280. Feinerman O, Veiga J, Dorfman JR, et al. Variability and robustness in T cell activation from regulated heterogeneity in protein levels. *Science*. 2008;321:1081-1084.
281. Das J, Ho M, Zikherman J, et al. Digital signaling and hysteresis characterize ras activation in lymphoid cells. *Cell*. 2009;136:337-351.
282. Feinerman O, Jentsch G, Tkach KE, et al. Single-cell quantification of IL-2 response by effector and regulatory T cells reveals critical plasticity in immune response. *Mol Syst Biol*. 2010;6:437.
283. Eldar A, Elowitz MB. Functional roles for noise in genetic circuits. *Nature*. 2010;467:167-173.
284. Neves SR. Modeling of spatially-restricted intracellular signaling. *Wiley Interdiscip Rev Syst Biol Med*. 2012;4:103-115.
285. Harris LA, Hogg JS, Faeder JR. Compartmental rule-based modeling of biochemical systems. *Proceedings of the 2009 Winter Simulation Conference*. Austin, TX; 2009:908-919.
286. Moraru II , Schaff JC, Slepchenko BM, et al. Virtual cell modelling and simulation software environment. *IET Syst Biol*. 2008;2:352-362.
287. Czech J, Dittrich M, Stiles JR. Rapid creation, Monte Carlo simulation, and visualization of realistic 3D cell models. *Methods Mol Biol*. 2009;500:237-287.
288. Andrews SS, Addy NJ, Brent R, et al. Detailed simulations of cell biology with Smoldyn 2.1. *PLoS Comput Biol*. 2010;6:e1000705.
289. Takahashi K, Tanase-Nicola S, Ten Wolde PR. Spatio-temporal correlations can drastically change the response of a MAPK pathway. *Proc Natl Acad Sci U S A*. 2010;107:2473-2478.
290. Walker DC, Southgate J. The virtual cell—a candidate co-ordinator for "middle-out" modelling of biological systems. *Brief Bioinform*. 2009;10:450-461.
291. Maus C, Rybacki S, Uhrmacher AM. Rule-based multi-level modeling of cell biological systems. *BMC Syst Biol*. 2011;5:166.
292. Pitt-Francis J, Pathmanathan P, Bernabeu MO, et al. Chaste: A test-driven approach to software development for biological modelling. *Comput Phys Commun*. 2009;180:2452-2471.
293. Karr JR, Sanghvi JC, Macklin DN, et al. A whole-cell computational model predicts phenotype from genotype. *Cell*. 2012;150:389-401.
295. Ali HR, Dawson SJ, Blows FM, et al. Aurora kinase A outperforms Ki67 as a prognostic marker in ER-positive breast cancer. *Br J Cancer*. 2012;106:1798-1806.
297. Bebenek M, Dus D, Kozlak J. Fas and Fas ligand as prognostic factors in human breast carcinoma. *Med Sci Monit*. 2006;12:CR457-CR461.
298. McCarthy MM, Sznol M, DiVito KA, et al. Evaluating the expression and prognostic value of TRAIL-R1 and TRAIL-R2 in breast cancer. *Clin Cancer Res*. 2005;11:5188-5194.
299. Vakkala M, Paakko P, Soini Y. Expression of caspases 3, 6 and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion. *Br J Cancer*. 1999;81:592-599.
300. Schmitz KJ, Grabellus F, Callies R, et al. Relationship and prognostic significance of phospho-(serine 166)-murine double minute 2 and Akt activation in node-negative breast cancer with regard to p53 expression. *Virchows Arch*. 2006;448:16-23.

301. Yamashita S, Masuda Y, Kurizaki T, et al. Survivin expression predicts early recurrence in early-stage breast cancer. *Anticancer Res*. 2007;27:2803-2808.
302. O'Driscoll L, Cronin D, Kennedy SM, et al. Expression and prognostic relevance of Mcl-1 in breast cancer. *Anticancer Res*. 2004;24:473-482.
303. Trere D, Montanaro L, Ceccarelli C, et al. Prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in human infiltrating ductal carcinomas of the breast. *Ann Oncol*. 2007;18:1004-1014.
306. Xia W, Chen JS, Zhou X, et al. Phosphorylation/cytoplasmic localization of p21Cip1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients. *Clin Cancer Res*. 2004;10:3815-3824.
311. Harigopal M, Berger AJ, Camp RL, et al. Automated quantitative analysis of E-cadherin expression in lymph node metastases is predictive of survival in invasive ductal breast cancer. *Clin Cancer Res*. 2005;11:4083-4089.
312. Dolled-Filhart M, McCabe A, Giltnane J, et al. Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer Res*. 2006;66:5487-5494.
314. Bircan S, Kapucuoglu N, Baspinar S, et al. CD24 expression in ductal carcinoma in situ and invasive ductal carcinoma of breast: an immunohistochemistry-based pilot study. *Pathol Res Pract*. 2006;202:569-576.
315. Morohashi S, Kusumi T, Sato F, et al. Decreased expression of claudin-1 correlates with recurrence status in breast cancer. *Int J Mol Med*. 2007;20:139-143.
316. Salvucci O, Bouchard A, Baccarelli A, et al. The role of CXCR4 receptor expression in breast cancer: a large tissue microarray study. *Breast Cancer Res Treat*. 2006;97:275-283.
317. Gilcrease MZ, Kilpatrick SK, Woodward WA, et al. Coexpression of alpha-6beta4 integrin and guanine nucleotide exchange factor Net1 identifies node-positive breast cancer patients at high risk for distant metastasis. *Cancer Epidemiol Biomarkers Prev*. 2009;18:80-86.
318. Kamat AA, Fletcher M, Gruman LM, et al. The clinical relevance of stromal matrix metalloproteinase expression in ovarian cancer. *Clin Cancer Res*. 2006;12:1707-1714.
319. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Flt-4-positive vessel density correlates with vascular endothelial growth factor-d expression, nodal status, and prognosis in breast cancer. *Clin Cancer Res*. 2003;9:5313-5317.
320. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Prognostic significance of vascular endothelial growth factor D in breast carcinoma with long-term follow-up. *Clin Cancer Res*. 2003;9:716-721.
321. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Clinicopathological significance of vascular endothelial growth factor-C in breast carcinoma with long-term follow-up. *Mod Pathol*. 2003;16:309-314.
322. Chen HH, Su WC, Lin PW, et al. Hypoxia-inducible factor-1alpha correlates with MET and metastasis in node-negative breast cancer. *Breast Cancer Res Treat*. 2007;103:167-175.
323. Cooke VG, LeBleu VS, Keskin D, et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell*. 2012;21:66-81.
325. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia*. 2006;8:190-198.
326. Tsutsui S, Yasuda K, Suzuki K, et al. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep*. 2005;14:425-431.
327. Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. *Cytokine Growth Factor Rev*. 2006;17:325-337.
328. Zerkowski MP, Camp RL, Burtness BA, et al. Quantitative analysis of breast cancer tissue microarrays shows high cox-2 expression is associated with poor outcome. *Cancer Invest*. 2007;25:19-26.
329. Kluger HM, Dolled-Filhart M, Rodov S, et al. Macrophage colony-stimulating factor-1 receptor expression is associated with poor outcome in breast cancer by large cohort tissue microarray analysis. *Clin Cancer Res*. 2004;10:173-177.
330. Henry LR, Lee HO, Lee JS, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. *Clin Cancer Res*. 2007;13:1736-1741.
331. Paulsson J, Sjoblom T, Micke P, et al. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. *Am J Pathol*. 2009;175:334-341.
332. Barroso MM. Quantum dots in cell biology. *J Histochem Cytochem*. 2011;59:237-251.
333. van der Krogt GNM, Ogink J, Ponsioen B, et al. A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. *PLoS One*. 2008;3:e1916.
334. Day RN, Davidson MW. Fluorescent proteins for FRET microscopy: monitoring protein interactions in living cells. *BioEssays*. 2012;34:341-350.
335. McNamara G, Gupta A, Reynaert J, et al. Spectral imaging microscopy web sites and data. *Cytometry A*. 2006;69:863-871.
336. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248-1250.
337. Chatr-aryamontri A, Breitkreutz B-J, Heinicke S, et al. The BioGRID interaction database: 2013 update. *Nucl Acids Res*. 2013;41:D816-D823.
338. Fernçndez-Suçrez XM, Galperin MY. The 2013 Nucleic Acids Research Database Issue and the online Molecular Biology Database Collection. *Nucl Acids Res*. 2013;41:D1-D7.
339. Orloff DN, Iwasa JH, Martone ME, et al. The cell: an image library-CCDB: a curated repository of microscopy data. *Nucl Acids Res*. 2013;41:D1241-D1250.
340. Krupp M, Itzel T, Maass T, et al. CellLineNavigator: a workbench for cancer cell line analysis. *Nucl Acids Res*. 2013;41:D942-D948.
341. Conlon S, Colgan A, Allen D, et al. Comparison of dual-colour chromogenic in-situ hybridization and fluorescence in-situ hybridization in the assessment of HER2 gene amplification in breast carcinoma. *Histopathology*. 2011;58:319-321.
342. Arnould L, Denoux Y, MacGrogan G, et al. Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. *Br J Cancer*. 2003;88:1587-1591.
343. Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. *Hum Pathol*. 2007;38:1105-1122.
344. Gupta D, Middleton LP, Whitaker MJ, et al. Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. *Am J Clin Pathol*. 2003;119:381-387.
345. Van Laere SJ, Van der Auwera I, Van den Eynden GG, et al. Nuclear factor-kappaB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-kappaB DNA-binding. *Clin Cancer Res*. 2006;12:3249-3256.
346. Biswas DK, Shi Q, Baily S, et al. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci U S A*. 2004;101:10137-10142.
347. Kielhorn E, Provost E, Olsen D, et al. Tissue microarray-based analysis shows phospho-beta-catenin expression in malignant melanoma is associated with poor outcome. *Int J Cancer*. 2003;103:652-656.
348. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
349. Verghese ET, Shenoy H, Cookson VJ, et al. Epithelial-mesenchymal interactions in breast cancer: evidence for a role of nuclear localized beta-catenin in carcinoma-associated fibroblasts. *Histopathology*. 2011;59:609-618.
351. Nevalainen MT, Xie J, Torhorst J, et al. Signal transducer and activator of transcription-5 activation and breast cancer prognosis. *J Clin Oncol*. 2004;22:2053-2060.
357. Habashy HO, Rakha EA, Aleskandarany M, et al. FOXO3a nuclear localisation is associated with good prognosis in luminal-like breast cancer. *Breast Cancer Res Treat*. 2011;129:11-21.

References

1. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.
2. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
3. Koboldt DC, Fulton RS, McLellan MD, et al. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490:61-70.
4. Gatza ML, Kung HN, Blackwell KL, et al. Analysis of tumor environmental response and oncogenic pathway activation identifies distinct basal and luminal features in HER2-related breast tumor subtypes. *Breast Cancer Res*. 2011;13:R62.
5. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005;121:335-348.
6. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;449:557-563.
7. Critchley-Thorne RJ, Yu HX, Lee PP. Immune signatures associated with the cancer bearing state. In: Marincola FM, Wang E, eds. *Signatures of Rejection*. New York, NY: Springer; 2010.
8. Chouaib S, Kieda C, Benlalam H, et al. Endothelial cells as key determinants of the tumor microenvironment: interaction with tumor cells, extracellular matrix and immune killer cells. *Crit Rev Immunol*. 2010;30:529-545.
9. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res*. 2010;316:1324-1331.
10. Ostrand-Rosenberg S, Sinha P, Beury DW, et al. Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression. *Semin Cancer Biol*. 2012;22:275-281.
11. Chen J, Yao Y, Gong C, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer Cell*. 2011;19:541-555.
12. Mantovani A, Savino B, Locati M, et al. The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev*. 2010;21:27-39.
13. Staveley-O'Carroll K, Sotomayor E, Montgomery J, et al. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci U S A*. 1998;95:1178-1183.
14. Sotomayor EM, Borrello I, Rattis FM, et al. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood*. 2001;98:1070-1077.
15. Whiteside TL. Tumor-induced death of immune cells: its mechanisms and consequences. *Semin Cancer Biol*. 2002;12:43-50.
16. Critchley-Thorne RJ, Miller SM, Taylor DL, et al. Applications of cellular systems biology in breast cancer patient stratification and diagnostics. *Comb Chem High Throughput Screen*. 2009;12:860-869.
17. Buchser WJ, Laskow TC, Pavlik PJ, et al. Cell-mediated autophagy promotes cancer cell survival. *Cancer Res*. 2012;72:2970-2979.
18. Albeck JG, Burke JM, Aldridge BB, et al. Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. *Mol Cell*. 2008;30:11-25.
19. Cohen AA, Geva-Zatorsky N, Eden E, et al. Dynamic proteomics of individual cancer cells in response to a drug. *Science*. 2008;322:1511-1516.
20. Abraham VC, Taylor DL, Haskins JR. High-content screening applied to large-scale cell biology. *Trends Biotechnol*. 2003;22:15-22.
21. Giuliano KA, Chen Y-T, Taylor DL. High-content screening with siRNA optimizes a cell biological approach to drug discovery: defining the role of p53 activation in the cellular response to anticancer drugs. *J Biomol Screen*. 2004;9:557-568.
22. Benjamin ER, Pruthi F, Olanrewaju S, et al. Pharmacological characterization of recombinant N-type calcium channel (Cav2.2) mediated calcium mobilization using FLIPR. *Biochem Pharmacol*. 2006;72:770-782.
23. Giuliano KA, Cheung WS, Curran DP, et al. Systems cell biology knowledge created from high content screening. *Assay Drug Dev Technol*. 2005;3:501-514.
24. Shibue T, Brooks MW, Inan MF, et al. The outgrowth of micrometastases is enabled by the formation of filopodium-like protrusions. *Cancer Discov*. 2012;2:706-721.
25. Shi J, Orth JD, Mitchison T. Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. *Cancer Res*. 2008;68:3269-3276.
26. Evensen L, Micklem DR, Link W, et al. A novel imaging-based high-throughput screening approach to anti-angiogenic drug discovery. *Cytometry A*. 2010;77:41-51.
27. Gupta PB, Fillmore CM, Jiang G, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell*. 2011;146:633-644.
28. Beliveau A, Mott JD, Lo A, et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. *Genes Dev*. 2010;24:2800-2811.
29. Yates C, Shepard CR, Papworth G, et al. Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. In: Vande Woude G, Klein G, eds. *Advances in Cancer Research: Applications of Viruses for Cancer Therapy*. Salt Lake City, Utah: Academic Press; 2007:225-246.
30. Galbraith W, Wagner MC, Chao J, et al. Imaging cytometry by multiparameter fluorescence. *Cytometry*. 1991;12:579-596.
31. Zal T, Chodaczek G. Intravital imaging of anti-tumor immune response and the tumor microenvironment. *Semin Immunopathol*. 2010;32:305-317.
32. Provenzano PP, Eliceiri KW, Keely PJ. Multiphoton microscopy and fluorescence lifetime imaging microscopy (FLIM) to monitor metastasis and the tumor microenvironment. *Clin Exp Metastasis*. 2009;26:357-370.
33. Niesner RA, Hauser AE. Recent advances in dynamic intravital multi-photon microscopy. *Cytometry A*. 2011;79A:789-798.
34. Boissonnas A, Fetler L, Zeelenberg IS, et al. In vivo imaging of cytotoxic T cell infiltration and elimination of a solid tumor. *J Exp Med*. 2007;204:345-356.
35. Alexander S, Koehl GE, Hirschberg M, et al. Dynamic imaging of cancer growth and invasion: a modified skin-fold chamber model 11. *Histochem Cell Biol*. 2008;130:1147-1154.
36. Koehl G, Gaumann A, Geissler EK. Intravital microscopy of tumor angiogenesis and regression in the dorsal skin fold chamber: mechanistic insights and preclinical testing of therapeutic strategies. *Clin Exp Metastasis*. 2009;26:329-344.
37. Kedrin D, Wyckoff J, Sahai E, et al. Imaging tumor cell movement in vivo. *Curr Protoc Cell Biol*. 2007;19:7.
38. Kedrin D, Gligorijevic B, Wyckoff J, et al. Intravital imaging of metastatic behavior through a mammary imaging window. *Nat Methods*. 2008;5:1019-1021.
39. Lang P, Yeow K, Nichols A, et al. Cellular imaging in drug discovery. *Nat Rev Drug Discov*. 2006;5:343-356.
40. Hoelder S, Clarke PA, Workman P. Discovery of small molecule cancer drugs: successes, challenges and opportunities. *Mol Oncol*. 2012;6:155-176.
41. Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? *Br J Pharmacol*. 2010;160:204-216.
42. Pereira C, Coutinho I, Soares J, et al. New insights into cancer-related proteins provided by the yeast model. *FEBS J*. 2012;279:697-712.
43. Barberis A, Gunde T, Berset C, et al. Yeast as a screening tool. *Drug Discov Today*. 2005;2:187-192.
44. Ohnuki S, Oka S, Nogami S, et al. High-content, image-based screening for drug targets in yeast. *PLoS One*. 2010;5:e10177.
45. Vizeacoumar F, van Dyk N, Vizeacoumar FS, et al. Integrating high-throughput genetic interaction mapping and high-content screening to explore yeast spindle morphogenesis. *J Cell Biol*. 2010;188:69-81.
46. Siddiqui SS, Loganathan S, Krishnaswamy S, et al. *C. elegans* as a model organism for in vivo screening in cancer: effects of human c-Met in lung cancer affect *C. elegans* vulva phenotypes. *Cancer Biol Ther*. 2008;7:856-863.
47. Bae Y-K, Sung JY, Kim Y-N, et al. An in vivo *C. elegans* model system for screening EGFR-inhibiting anti-cancer drugs. *PLoS One*. 2012;7:1-9.
48. Matus DQ, Li X-Y, Durbin S, et al. In vivo identification of regulators of cell invasion across basement membranes. *Sci Signal*. 2010;3:1-9.
49. Rudrapatna VA, Cagan RL, Das TK. Drosophila cancer models. *Dev Dynam*. 2012;241:1-12.
50. Miles WO, Dyson NJ, Walker JA. Modeling tumor invasion and metastasis in Drosophila. *Dis Models Mech*. 2011;4:753-761.
51. Chen C-L, Schroeder MC, Kango-Singh M, et al. Tumor suppression by cell competition through regulation of the Hippo pathway. *Proc Natl Acad Sci U S A*. 2012;109:484-489.

52. Dar AC, Das TK, Shokat KM, et al. Chemical genetic discovery of targets and anti-targets for cancer polypharmacology. *Nature*. 2012;486:80-85.
53. Binder V, Zon LI. High throughput in vivo phenotyping: the zebrafish as a tool for drug discovery for hematopoietic stem cells and cancer. 10.1016/j.ddmod.2012.02.007.
54. Feitsma H, Cuppen E. Zebrafish as a cancer model. *Mol Cancer Res*. 2008;6:685-694.
55. Taylor AM, Zon LI. Zebrafish tumor assays: the state of transplantation. *Zebrafish*. 2009;6:339-346.
56. Vogt A, Cholewinski A, Shen X, et al. Automated image-based phenotypic analysis in zebrafish embryos. *Dev Dynam*. 2009;238:656-663.
57. Prescher JA, Contag CH. Guided by the light: visualizing biomolecular processes in living animals with bioluminescence. *Curr Opin Chem Biol*. 2010;14:80-89.
58. Pittet MJ, Weissleder R. Intravital imaging. *Cell*. 2011;147:983-991.
59. Croix CS, Zipfel WR, Watkins SC. Potential solutions for confocal imaging of living animals. *Biotechniques*. 2007;43:14-19.
60. Kaijzel EL, van der Pluijm G, Löwen Clemens WGM. Whole-body optical imaging in animal models to assess cancer development and progression. *Clin Cancer Res*. 2007;13:3490-3497.
61. Giuliano K, DeBiasio R, Dunlay RT, et al. High content screening: a new approach to easing key bottlenecks in the drug discovery process. *J Biomol Screen*. 1997;2:249-259.
62. Giuliano KA, Haskins JR, Taylor DL. Advances in high content screening for drug discovery. *Assays Drug Dev Tech*. 2003;1:565-577.
63. Giuliano KA, Taylor DL, Waggoner AS. Reagents to measure and manipulate cell functions. In: *High Content Screening*. Totowa, NJ: Humana Press; 2006:141-163.
64. Taylor DL. Past, present, and future of high content screening and the field of cellomics. *Methods Mol Biol*. 2007;356:3-18.
65. Haney SA, ed. *High Content Screening: Science, Techniques, and Applications*. New York, NY: Wiley; 2008.
66. Inglese J, ed. *Methods in Enzymology: Vol 414. Measuring Biological Responses with Automated Microscopy*. New York, NY: Academic Press; 2006.
67. Taylor D, Haskins J, Giuliano K, eds. *Methods in Molecular Biology: Vol 356. High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery*. New York, NY: Springer; 2007.
68. Bullen A. Microscopic imaging techniques for drug discovery. *Nat Rev Drug Discov*. 2008;7:54-67.
69. Thomas N. High-content screening: a decade of evolution. *J Biomol Screen*. 2010;15:1-7.
70. Critchley-Thorne RJ, Miller SM, Taylor DL, et al. Applications of cellular systems biology in breast cancer patient stratification and diagnostics. *Comb Chem High Throughput Screen*. 2009;12:860-869.
71. Zanella F, Lorens JB, Link W. High content screening: seeing is believing. *Trends Biotechnol*. 2010;28:237-245.
72. Zock J. Applications of high content screening in life science research. *Comb Chem High Throughput Screen*. 2009;12:870-876.
73. Purdue University Cytometry Laboratory. *Antibodies, Reagents, Fluorescent Probes*. Retrieved from http://www.cyto.purdue.edu/flowcyt/supplier/reagents.htm.
74. Salk Institute for Biological Studies. *Table of Fluorochromes*. Retrieved from http://flowcyt.salk.edu/fluo.html.
75. Spence MTZ, Johnson ID. *The Molecular Probes Handbook: a Guide to Fluorescent Probes and Labeling Technologies*. 11th ed. Carlsbad, CA: Life Technologies Corp; 2010.
76. Fleuret F, Li T, Dubout C, et al. Comparing machines and humans on a visual categorization test. *Proc Natl Acad Sci U S A*. 2011;108:17621-17625.
77. Wood KC, Konieczkowski DJ, Johannessen CM, et al. MicroSCALE screening reveals genetic modifiers of therapeutic response in melanoma. *Sci Signal*. 2012;5:1-12.
78. Cheong R, Paliwal S, Levchenko A. High content screening in microfluidic devices. *Expert Opin Drug Discov*. 2010;5:715-720.
79. Fuchs F, Pau G, Kranz D, et al. Clustering phenotype populations by genome-wide RNAi and multiparametric imaging. *Mol Syst Biol*. 2010;6:370.
80. Murphy RF. An active role for machine learning in drug development. *Nat Chem Biol*. 2011;7:327-330.
81. Sasaki K, Ito T, Nishino N. Real-time imaging of histone H4 hyperacetylation in living cells. *Proc Natl Acad Sci U S A*. 2009;106:16257-16262.
82. Ashall L, Horton CA, Nelson DE. Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science*. 2009;324:242-246.
83. Kalén M, Wallgard E, Asker N. Combination of reverse and chemical genetic screens reveals angiogenesis inhibitors and targets. *Chem Biol*. 2009;16:432-441.
84. Livesey KM, Kang R, Vernon P, et al. p53/HMGB1 complexes regulate autophagy and apoptosis. *Cancer Res*. 2012;72:1996-2005.
85. Norton J, Titus SA, Dexter D. Automated high content screening for compounds that disassemble the perinucleolar compartment. *J Biomol Screen*. 2009;14:1045-1053.
86. Raccor B, Vogt A, Sikorski RP. Cell-based and biochemical structure-activity analyses of analogs of the microtubule stabilizer dictyostatin. *Mol Pharmacol*. 2008;73:718-726.
87. Conrad C, Gerlich DW. Automated microscopy for high-content RNAi screening. *J Cell Biol*. 2010;188:453-461.
88. Moffat J, Sabatini DM. Building mammalian signalling pathways with RNAi screens. *Nat Rev Mol Cell Biol*. 2006;7:177-187.
89. Rines D, Gomez-Ferreria MA, Zhou Y. Whole genome functional analysis identifies novel components required for mitotic spindle integrity in human cells. *Genome Biol*. 2008;9:R44.
90. Antczak C, Takagi T, Ramirez CN, et al. Live-cell imaging of caspase activation for high-content screening. *J Biomol Screen*. 2009;14:956-969.
91. Guzi T, Paruch K, Dwyer MP, et al. Targeting the replication checkpoint using SCH 900776, a potent and functionally selective CHK1 inhibitor identified via high content screening. *Mol Cancer Ther*. 2011;10:591-602.
92. Neumann B, Walter T, Hériché JK. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature*. 2010;464:721-727.
93. Schmitz M, Held M, Janssens V, et al. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells 8. *Nat Cell Biol*. 2010;12:886-893.
94. Mayer TU, Kapoor TM, Haggarty SJ, et al. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science*. 1999;286:971-974.
95. Haney S, LaPan P, Pan J, et al. High content screening moves to the front of the line. *Drug Discov Today*. 2006;11:889-894.
96. Bickle M. The beautiful cell: high-content screening in drug discovery 2. *Anal Bioanal Chem*. 2010;398:219-226.
97. Birsoy K, Sabatini DM, Possemato R. Targeting cancer metabolism: a bedside lesson. *Nat Med*. 2012;18:1022-1023.
98. Arora R, Shuda M, Gustafierro A, et al. Viral knockdown reveals surviving as a therapeutic target for Merkel cell carcinoma. *Science Transl Med*. 2012;4:133ra56.
99. Xu G, Mawji I, Macrae C, et al. A high-content chemical screen identifies ellipticine as a modulator of p53 nuclear localization. *Apoptosis*. 2008;13:413-422.
100. Molina G, Vogt A, Bakan A, et al. Zebrafish chemical screening reveals an inhibitor of Dusp6 that expands cardiac cell lineages. *Nat Chem Biol*. 2009;5:680-687.
101. Danli L, Nicholl EE, Comess KM, et al. Development of a high-content screening assay panel to accelerate mechanism of action studies for oncology research. *J Biomol Screen*. 2012;17:1005-1017.
102. Anagnostou VK, Dimou AT, Botsis T, et al. Molecular classification of nonsmall cell lung cancer using a 4-protein quantitative assay. *Cancer*. 2012;118:1607-1618.
103. Link W, Oyarzabal J, Serelde BG. Chemical interrogation of FOXO3a nuclear translocation identifies potent and selective inhibitors of phosphoinositide 3-kinases. *J Biol Chem*. 2009;284:28392-28400.
104. Zanella F, Rosado A, García B. Chemical genetic analysis of FOXO nuclear-cytoplasmic shuttling by using image-based cell screening. *Chembiochem*. 2008;9:2229-2237.
105. Loo L-H, Wu LF, Altschuler SJ. Image-based multivariate profiling of drug responses from single cells. *Nat Methods*. 2007;4:445-453.
106. Perlman ZE, Slack MD, Feng Y, et al. Multidimensional drug profiling by automated microscopy. *Science*. 2004;306:1194-1198.
107. Schreiber S, Kapoor TM, Wess G. *Chemical Biology: From Small Molecules to Systems Biology and Drug Design*. Weinheim, Germany: Wiley-VCH; 2007.

108. DeBiasio R, Bright GR, Ernst LA, et al. Five parameter fluorescence imaging: wound healing of living Swiss 3T3 cells. *J Cell Biol*. 1987;105:1613-1622.
109. Sachs K, Perez O, Pe'er D, et al. Causal protein-signaling networks derived from multiparameter single-cell data. *Science*. 2005;308:523-529.
110. Xing Y, Chaudry Q, Shen C, et al. Bioconjugated quantum dots for multiplexed and quantitative immunohistochemistry. *Nat Protoc*. 2007;2:1152-1165.
111. Barash E, Dinn S, Sevinsky C, et al. Multiplexed analysis of proteins in tissue using multispectral fluorescence imaging. *IEEE Trans Med Imaging*. 2010;29:1457-1462.
112. Mittag A, Lenz D, Bocsi J, et al. Sequential photobleaching of fluorochromes for polychromatic slide-based cytometry. *Cytometry A*. 2006;69:139-141.
113. Wahlby C, Erlandsson F, Bengtsson E, et al. Sequential immunofluorescence staining and image analysis for detection of large numbers of antigens in individual cell nuclei. *Cytometry*. 2002;47:32-41.
114. Schubert W, Bonnekoh B, Pommer AJ, et al. Analyzing proteome topology and function by automated multidimensional fluorescence microscopy. *Nat Biotechnol*. 2006;24:1270-1278.
115. Schubert W, Gieseler A, Krusche A, et al. Next-generation biomarkers based on 100-parameter functional super-resolution microscopy TIS. *N Biotechnol*. 2012;29:599-610.
116. Gerdes MJ, Sevinsky CJ, Sood A, et al. Highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. *Proc Natl Acad Sci U S A*. 2013;110:11982-11987.
117. Esposito A, Wouters FS. Fluorescence lifetime imaging microscopy. *Curr Protoc Cell Biol*. 2004 4.14.
118. Setou M. Imaging Mass Spectrometry: Protocols for Mass Spectrometry. New York: Springer, 2010
119. Riemann B, Schafers KP, Schober O, et al. Small animal PET in preclinical studies: opportunities and challenges. *Q J Nucl Med Mol Imaging*. 2008;52:215-221.
120. Vande Velde G, Baekelandt V, Dresselaers T. Magnetic resonance imaging and spectroscopy methods for molecular imaging. *Q J Nucl Med Mol Imaging*. 2009;53:565-585.
121. Kraljevic S, Sedic M, Scott M, et al. Casting light on molecular events underlying anti-cancer drug treatment: what can be seen from the proteomics point of view? *Cancer Treat Rev*. 2006;32:619-629.
122. Turner NC, Reis-Filho JS. Genetic heterogeneity and cancer drug resistance. *Lancet Oncol*. 2012;13:e178-e185.
123. Niepel M, Spencer SL, Sorger PK. Non-genetic cell-to-cell variability and the consequences for pharmacology. *Curr Opin Chem Biol*. 2009;13:556-561.
124. Spencer SL, Gaudet S, Albeck JG, et al. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature*. 2009;459:428-432.
125. Huang S. Non-genetic heterogeneity of cells in development: more than just noise. *Development*. 2009;136:3853-3862.
126. Bhadriraju K, Chen CS. Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov Today*. 2002;7:612-620.
127. Wang D, Bodovitz S. Single cell analysis: the new frontier in "omics". *Trends Biotechnol*. 2010;28:281-290.
128. Towne DL, Nicholl EE, Comess KM, et al. Development of a high-content screening assay panel to accelerate mechanism of action studies for oncology research. *J Biomol Screen*. 2012;17:1005-1017.
129. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat*. 2004;83:249-289.
130. Clark MJ, Homer N, O'Connor BD, et al. U87MG decoded: the genomic sequence of a cytogenetically aberrant human cancer cell line. *PLoS Genet*. 2010;6:e1000832.
131. Borrell B. How accurate are cancer cell lines? *Nature*. 2010;463:858.
132. Gillet J-P, Calcagno AM, Varma S, et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proc Natl Acad Sci U S A*. 2011;108:18708-18713.
133. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature*. 2012;483:603-307.
134. Neve RM, Chin K, Fridlyand J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. 2006;10:515-527.
135. Weinstein JN. Drug discovery: cell lines battle cancer. *Nature*. 2012;483:544-545.
136. Reinhold WC, Sunshine M, Liu H, et al. CellMiner: a web-based suite of genomic and pharmacologic tools to explore transcript and drug patterns in the NCI-60 cell line set. *Cancer Res*. 2012;72:3499-3511.
137. Liu H, D'Andrade P, Fulmer-Smentek S, et al. mRNA and microRNA expression profiles of the NCI-60 integrated with drug activities. *Mol Cancer Ther*. 2010;9:1080-1091.
138. National Cancer Institute. *The Cancer Genome Atlas*. Retrieved from http://cancergenome.nih.gov/publications.
139. Pandita A, Aldape KD, Zadeh G, et al. Contrasting in vivo and in vitro fates of glioblastoma cell subpopulations with amplified EGFR. *Genes Chromosomes Cancer*. 2004;39:29-36.
140. Keller PJ, Lin AF, Arendt LM, et al. Mapping the cellular and molecular heterogeneity of normal and malignant breast tissues and cultured cell lines. *Breast Cancer Res*. 2010;12:R87.
141. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res*. 1997;57:3071-3078.
142. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009;4:e6146.
143. Giuliano KA, Johnston PA, Gough A, et al. Systems cell biology based on high-content screening. *Methods Enzymol*. 2006;414:601-619.
144. Kim H, Phung Y, Ho M. Changes in global gene expression associated with 3D structure of tumors: an ex vivo matrix-free mesothelioma spheroid model. *PLoS One*. 2012;7:e39556.
145. Kunz-Schughart LA, Freyer JP, Hofstaedter F, et al. The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. *J Biomol Screen*. 2004;9:273-285.
146. Kenny PA, Lee GY, Myers CA, et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol*. 2007;1:84-96.
147. Gautrot JE, Wang C, Liu X, et al. Mimicking normal tissue architecture and perturbation in cancer with engineered micro-epidermis. *Biomaterials*. 2012;33:5221-5229.
148. Pinto MP, Jacobsen BM, Horwitz KB. An immunohistochemical method to study breast cancer cell subpopulations and their growth regulation by hormones in three-dimensional cultures. *Front Endocrinol (Lausanne)*. 2011;2:15.
149. Leung CT, Brugge JS. Outgrowth of single oncogene-expressing cells from suppressive epithelial environments. *Nature*. 2012;482:410-413.
150. Norton KA, Namazi S, Barnard N, et al. Automated reconstruction algorithm for identification of 3D architectures of cribriform ductal carcinoma in situ. *PLoS One*. 2012;7:e44011.
151. Caicedo-Carvajal CE, Liu Q, Remache Y, et al. Cancer tissue engineering: a novel 3D polystyrene scaffold for in vitro isolation and amplification of lymphoma cancer cells from heterogeneous cell mixtures. *J Tissue Eng*. 2011;2011:362326.
152. Dhiman HK, Ray AR, Panda AK. Three-dimensional chitosan scaffold-based MCF-7 cell culture for the determination of the cytotoxicity of tamoxifen. *Biomaterials*. 2005;26:979-986.
153. Dutta RC, Dutta AK. Cell-interactive 3D-scaffold; advances and applications. *Biotechnol Adv*. 2009;27:334-339.
154. Dutta RC. In search of optimal scaffold for regenerative medicine and therapeutic delivery. *Ther Deliv*. 2011;2:231-234.
155. Timpson P, McGhee EJ, Anderson KI. Imaging molecular dynamics in vivo—from cell biology to animal models. *J Cell Sci*. 2011;124:2877-2890.
156. Gil V, del Rio JA. Analysis of axonal growth and cell migration in 3D hydrogel cultures of embryonic mouse CNS tissue. *Nat Protoc*. 2012;7:268-280.
157. Ziolkowska K, Kwapiszewski R, Brzozka Z. Microfluidic devices as tools for mimicking the in vivo environment. *N J Chem*. 2011;35:979-990.
158. Vinci M, Gowan S, Boxall F, et al. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC Biol*. 2012;10:29.
159. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res*. 2011;13:215.
160. Altschuler SJ, Wu LF. Cellular heterogeneity: do differences make a difference? *Cell*. 2010;141:559-563.
161. Merlo LMF, Pepper JW, Reid BJ, et al. Cancer as an evolutionary and ecological process. *Nat Rev Cancer*. 2006;6:924-935.

162. Dalerba P, Kalisky T, Sahoo D, et al. Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat Biotechnol*. 2011;29:1120-1127.
163. Houston MA, Augenlicht LH, Heerdt BG. Stable differences in intrinsic mitochondrial membrane potential of tumor cell subpopulations reflect phenotypic heterogeneity. *Int J Cell Biol*. 2011 2011: 978583.
164. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
165. Singh DK, Ku CJ, Wichaidit C, et al. Patterns of basal signaling heterogeneity can distinguish cellular populations with different drug sensitivities. *Mol Syst Biol*. 2010;6:369.
166. Feng Y, Mitchison TJ, Bender A, et al. Multi-parameter phenotypic profiling: using cellular effects to characterize small-molecule compounds. *Nat Rev Drug Discov*. 2009;8:567-578.
167. Giuliano KA, Gough AH, Taylor DL, et al. Early safety assessment using cellular systems biology yields insights into mechanisms of action. *J Biomol Screen*. 2010;15:783-797.
168. Kummel A, Selzer P, Siebert D, et al. Differentiation and visualization of diverse cellular phenotypic responses in primary high-content screening. *J Biomol Screen*. 2012;17:843-849.
169. Jones TR, Carpenter AE, Lamprecht MR, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci U S A*. 2009;106:1826-1831.
170. Ng AY, Rajapakse JC, Welsch RE, et al. A cell profiling framework for modeling drug responses from HCS imaging. *J Biomol Screen*. 2010;15:858-868.
171. Loo LH, Lin HJ, Steininger 3rd RJ, et al. An approach for extensibly profiling the molecular states of cellular subpopulations. *Nat Methods*. 2009;6:759-765.
172. Young IT. Proof without prejudice: use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. *J Histochem Cytochem*. 1977;25:935-941.
173. Kullback S. *Information Theory and Statistics*. Mineola, NY: Dover Publications; 1997.
174. Souissi I, Ladam P, Cognet JA, et al. A STAT3-inhibitory hairpin decoy oligodeoxynucleotide discriminates between STAT1 and STAT3 and induces death in a human colon carcinoma cell line. *Mol Cancer*. 2012;11:12.
175. Rexhepaj E, Brennan DJ, Holloway P, et al. Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: application to measurement of oestrogen and progesterone receptor levels in breast cancer. *Breast Cancer Res*. 2008;10:R89.
176. Sharangpani GM, Joshi AS, Porter K, et al. Semi-automated imaging system to quantitate estrogen and progesterone receptor immunoreactivity in human breast cancer. *J Microsc*. 2007;226:244-255.
177. Huang W, Hennrick K, Drew S. A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Hum Pathol*. 2013;44:29-38.
178. Chakravarti A, DeSilvio M, Zhang M, et al. Prognostic value of p16 in locally advanced prostate cancer: a study based on Radiation Therapy Oncology Group Protocol 9202. *J Clin Oncol*. 2007;25:3082-3089.
179. Nassar A, Cohen C, Agersborg SS, et al. A multisite performance study comparing the reading of immunohistochemical slides on a computer monitor with conventional manual microscopy for estrogen and progesterone receptor analysis. *Am J Clin Pathol*. 2011;135:461-467.
180. Nassar A, Cohen C, Agersborg SS, et al. A new immunohistochemical ER/PR image analysis system: a multisite performance study. *Appl Immunohistochem Mol Morphol*. 2011;19:195-202.
181. Jeung J, Patel R, Vila L, et al. Quantitation of HER2/neu expression in primary gastroesophageal adenocarcinomas using conventional light microscopy and quantitative image analysis. *Arch Pathol Lab Med*. 2012;136:610-617.
182. Taylor CR, Levenson RM. Quantification of immunohistochemistry—issues concerning methods, utility and semiquantitative assessment II. *Histopathology*. 2006;49:411-424.
183. Leong AS. Pitfalls in diagnostic immunohistology. *Adv Anat Pathol*. 2004;11:86-93.
184. Jain RK, Mehta R, Dimitrov R, et al. Atypical ductal hyperplasia: interobserver and intraobserver variability. *Mod Pathol*. 2011;24:917-923.
185. Castro NP, Osorio CA, Torres C, et al. Evidence that molecular changes in cells occur before morphological alterations during the progression of breast ductal carcinoma. *Breast Cancer Res*. 2008;10:R87.
186. Nederlof M, Watanabe S, Burnip B, et al. High-throughput profiling of tissue and tissue model microarrays: combined transmitted light and 3-color fluorescence digital pathology. *J Pathol Inform*. 2011;2:50.
187. Laurinavicius A, Laurinaviciene A, Ostapenko V, et al. Immunohistochemistry profiles of breast ductal carcinoma: factor analysis of digital image analysis data. *Diagn Pathol*. 2012;7:27.
188. Tadrous PJ. On the concept of objectivity in digital image analysis in pathology. *Pathology*. 2010;42:207-211.
189. Madabhushi A. Digital pathology image analysis: opportunities and challenges. *Imaging Med*. 2009;1:7-10.
190. Robertson D, Savage K, Reis-Filho JS, et al. Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue. *BMC Cell Biol*. 2008;9:13.
191. Donovan MJ, Khan FM, Bayer-Zubek V, et al. A systems-based modelling approach using transurethral resection of the prostate (TURP) specimens yielded incremental prognostic significance to Gleason when predicting long-term outcome in men with localized prostate cancer. *BJU Int*. 2012;109:207-213.
192. Wang J, Barnes RO, West NR, et al. Jab1 is a target of EGFR signaling in ERalpha-negative breast cancer. *Breast Cancer Res*. 2008;10:R51.
193. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med*. 2002;8:1323-1327.
194. Giltnane JM, Molinaro A, Cheng H, et al. Comparison of quantitative immunofluorescence with conventional methods for HER2/neu testing with respect to response to trastuzumab therapy in metastatic breast cancer. *Arch Pathol Lab Med*. 2008;132:1635-1647.
195. Bloom K, Harrington D. Enhanced accuracy and reliability of HER-2/neu immunohistochemical scoring using digital microscopy. *Am J Clin Pathol*. 2004;121:620-630.
196. Gustavson MD, Bourke-Martin B, Reilly D, et al. Standardization of HER2 immunohistochemistry in breast cancer by automated quantitative analysis. *Arch Pathol Lab Med*. 2009;133:1413-1419.
197. Sempere LF, Preis M, Yezefski T, et al. Fluorescence-based codetection with protein markers reveals distinct cellular compartments for altered microRNA expression in solid tumors. *Clin Cancer Res*. 2010;16:4246-4255.
198. Shachaf CM, Elchuri SV, Koh AL, et al. A novel method for detection of phosphorylation in single cells by surface enhanced Raman scattering (SERS) using composite organic-inorganic nanoparticles (COINs). *PLoS One*. 2009;4:e5206.
199. Bhattacharya S, Mathew G, Ruban E, et al. Toponome imaging system: in situ protein network mapping in normal and cancerous colon from the same patient reveals more than five-thousand cancer specific protein clusters and their subcellular annotation by using a three symbol code. *J Proteome Res*. 2012;9:6112-6125.
200. Fernandez DC, Bhargava R, Hewitt SM, et al. Infrared spectroscopic imaging for histopathologic recognition. *Nat Biotechnol*. 2005;23:469-474.
201. Freudiger CW, Min W, Saar BG, et al. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. *Science*. 2008;322:1857-1861.
202. Yang Y, Li F, Gao L, et al. Differential diagnosis of breast cancer using quantitative, label-free and molecular vibrational imaging. *Biomed Opt Express*. 2011;2:2160-2174.
203. Bird B, Miljkovic MS, Remiszewski S, et al. Infrared spectral histopathology (SHP): a novel diagnostic tool for the accurate classification of lung cancer. *Lab Invest*. 2012;92:1358-1373.
204. Kwak JT, Hewitt SM, Sinha S, et al. Multimodal microscopy for automated histologic analysis of prostate cancer. *BMC Cancer*. 2011;11:62.
205. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol*. 2007;25:5287-5312.
206. McCabe A, Dolled-Filhart M, Camp RL, et al. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. *J Natl Cancer Inst*. 2005;97:1808-1815.
207. Cuzick J, Dowsett M, Pineda S, et al. Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. *J Clin Oncol*. 2011;29:4273-4278.

208. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med*. 2004;351:2817-2826.
209. Paik S. Development and clinical utility of a 21-gene recurrence score prognostic assay in patients with early breast cancer treated with tamoxifen. *Oncologist*. 2007;12:631-635.
210. Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). *Clin Breast Cancer*. 2006;7:347-350.
211. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*. 2002;347:1999-2009.
212. Mook S, Van't Veer LJ, Rutgers EJ, et al. Individualization of therapy using Mammaprint: from development to the MINDACT Trial. *Cancer Genomics Proteomics*. 2007;4:147-155.
213. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008;14:518-527.
214. Van Laere SJ, Van der Auwera I, Van den Eynden GG, et al. NF-kappaB activation in inflammatory breast cancer is associated with oestrogen receptor downregulation, secondary to EGFR and/or ErbB2 overexpression and MAPK hyperactivation. *Br J Cancer*. 2007;97:659-669.
215. Dolled-Filhart M, Camp RL, Kowalski DP, et al. Tissue microarray analysis of signal transducers and activators of transcription 3 (Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. *Clin Cancer Res*. 2003;9:594-600.
216. Mefford D, Mefford J. Stromal genes add prognostic information to proliferation and histoclinical markers: a basis for the next generation of breast cancer gene signatures. *PLoS One*. 2012;7:e37646.
217. Robinson BD, Sica GL, Liu YF, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res*. 2009;15:2433-2441.
218. Ladanyi A, Kiss J, Somlai B, et al. Density of DC-LAMP$^+$ mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. *Cancer Immunol Immunother*. 2007;56:1459-1469.
219. Oktay MH, Gertler FB, Liu YF, et al. Correlated immunohistochemical and cytological assays for the prediction of hematogenous dissemination of breast cancer. *J Histochem Cytochem*. 2012;60:168-173.
220. Medrek C, Ponten F, Jirstrom K, et al. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer*. 2012;12:306.
221. Wolberg WH, Street WN. Computer-generated nuclear features compared with axillary lymph node status and tumor size as indicators of breast cancer survival. *Hum Pathol*. 2002;33:1086-1091.
222. Honeth G, Bendahl PO, Ringner M, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res*. 2008;10:R53.
223. Neumeister V, Agarwal S, Bordeaux J, et al. In situ identification of putative cancer stem cells by multiplexing ALDH1, CD44, and cytokeratin identifies breast cancer patients with poor prognosis. *Am J Pathol*. 2010;176:2131-2138.
224. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol*. 2006;24:5373-5380.
225. Weise A, Timmermann B, Grabherr M, et al. High-throughput sequencing of microdissected chromosomal regions. *Eur J Hum Genet*. 2010;18:457-462.
226. Cha S, Imielinski MB, Rejtar T, et al. In situ proteomic analysis of human breast cancer epithelial cells using laser capture microdissection: annotation by protein set enrichment analysis and gene ontology. *Mol Cell Proteomics*. 2010;9:2529-2544.
227. Finkelstein SD, Marsh W, Demetris AJ, et al. Microdissection-based allelotyping discriminates de novo tumor from intrahepatic spread in hepatocellular carcinoma. *Hepatology*. 2003;37:871-879.
228. Johann DJ, Rodriguez-Canales J, Mukherjee S, et al. Approaching solid tumor heterogeneity on a cellular basis by tissue proteomics using laser capture microdissection and biological mass spectrometry. *J Proteome Res*. 2009;8:2310-2318.
229. Finkelstein SD, Przygodzki R, Swalsky PA. Microdissection-based p53 genotyping: concepts for molecular testing. *Mol Diagn*. 1998;3:179-191.
230. Yang F, Foekens JA, Yu J, et al. Laser microdissection and microarray analysis of breast tumors reveal ER-alpha related genes and pathways. *Oncogene*. 2006;25:1413-1419.
231. Wittliff JL, Kruer TL, Andres SA, et al. Molecular signatures of estrogen receptor-associated genes in breast cancer predict clinical outcome. *Adv Exp Med Biol*. 2008;617:349-357.
232. Gregg JL, Brown KE, Mintz EM, et al. Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection. *BMC Cancer*. 2010;10:165.
233. Dietrich D, Lesche R, Tetzner R, et al. Analysis of DNA methylation of multiple genes in microdissected cells from formalin-fixed and paraffin-embedded tissues. *J Histochem Cytochem*. 2009;57:477-489.
234. Gagnon JF, Sanschagrin F, Jacob S, et al. Quantitative DNA methylation analysis of laser capture microdissected formalin-fixed and paraffin-embedded tissues. *Exp Mol Pathol*. 2010;88:184-189.
235. Morris MK, Saez-Rodriguez J, Clarke DC, et al. Training signaling pathway maps to biochemical data with constrained fuzzy logic: quantitative analysis of liver cell responses to inflammatory stimuli. *PLoS Comput Biol*. 2011;7:e1001099.
236. Nederlof MA, Witkin AP, Taylor DL. Knowledge-driven image analysis of cell structures. *Proc SPIE*. 1991;1428:233.
237. Nandy K, Gudla PR, Amundsen R, et al. Automatic segmentation and supervised learning-based selection of nuclei in cancer tissue images. *Cytometry A*. 2012;81:743-754.
238. Ginty F, Adak S, Can A, et al. The relative distribution of membranous and cytoplasmic met is a prognostic indicator in stage I and II colon cancer. *Clin Cancer Res*. 2008;14:3814-3822.
239. Baatz M, Arini N, Schape A, et al. Object-oriented image analysis for high content screening: detailed quantification of cells and sub cellular structures with the Cellenger software. *Cytometry A*. 2006;69:652-658.
240. Tredan O, Galmarini CM, Patel K, et al. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst*. 2007;99:1441-1454.
241. Chennubhotla C, Jepson A. EigenCuts: Half-lives of eigenflows for spectral clustering. *NIPS*. 2003;15:689-896.
242. Chennubhotla C, Jepson A. Hierarchical eigensolver for transition matrices in spectral methods. *NIPS*. 2005;17:273-280.
243. Burger V, Chennubhotla CNHS. network-based hierarchical segmentation for cryo-electron microscopy density maps. *Biopolymers*. 2012;97:732-741.
244. Mitchell T. Machine learning and data mining. *Commun ACM*. 1999;42:31-36.
245. DeRisi J, Penland L, Brown PO, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet*. 1996;14:457-460.
246. Zhang L, Zhou W, Velculescu VE, et al. Gene expression profiles in normal and cancer cells. *Science*. 1997;276:1268-1272.
247. Qu Y, Adam BL, Yasui Y, et al. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clin Chem*. 2002;48:1835-1843.
248. Su AI, Welsh JB, Sapinoso LM, et al. Molecular classification of human carcinomas by use of gene expression signatures. *Cancer Res*. 2001;61:7388-7393.
249. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *Nat Med*. 2003;9:416-423.
250. Meinel LA, Stolpen AH, Berbaum KS, et al. Breast MRI lesion classification: improved performance of human readers with a backpropagation neural network computer-aided diagnosis (CAD) system. *J Magn Reson Imaging*. 2007;25:89-95.
251. Beck AH, Sangoi AR, Leung S, et al. Systematic analysis of breast cancer morphology uncovers stromal features associated with survival. *Sci Transl Med*. 2011;3:108ra113.
252. Beenken SW, Grizzle WE, Crowe DR, et al. Molecular biomarkers for breast cancer prognosis: coexpression of c-erbB-2 and p53. *Ann Surg*. 2001;233:630-638.
253. Glory E, Newberg J, Murphy RF. Automated comparison of protein subcellular location patterns between images of normal and cancerous tissues. *Proc IEEE Int Symp Biomed Imaging*. 2008;4540993:304-307.
254. Ellison DW, Onilude OE, Lindsey JC, et al. beta-Catenin status predicts a favorable outcome in childhood medulloblastoma: the United Kingdom Children's Cancer Study Group Brain Tumour Committee. *J Clin Oncol*. 2005;23:7951-7957.
255. Alon U, Barkai N, Notterman DA, et al. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc Natl Acad Sci U S A*. 1999;96:6745-6750.

256. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*. 1999;286:531-537.
257. Chen X, Velliste M, Weinstein S, et al. Location proteomics—building subcellular location trees from high resolution 3D fluorescence microscope images of randomly-tagged proteins. *Proc SPIE*. 2003; 4962:298-306.
258. Slack MD, Martinez ED, Wu LF, et al. Characterizing heterogeneous cellular responses to perturbations. *Proc Natl Acad Sci U S A*. 2008;105: 19306-19311.
259. Giuliano KA, Premkumar D, Strock CJ, et al. Cellular systems biology profiling applied to cellular models of disease. *Comb Chem High Throughput Screen*. 2009;12:838-848.
260. Hample FR, Ronchetti EM, Rousseeuw PJ, et al. *Robust Statistics: The Approach Based on Influence Functions*. New York, NY: John Wiley and Sons; 1986.
261. Chung FRK. *Spectral graph theory*. *CBMS Lectures*. Providence, RI: American Mathematical Society; 1997.
262. McLachlan GJ. *Basford. KE. Mixture Models: Inference and Applications to Clustering*. New York, NY: Marcel Dekker; 1988.
263. Li C, Hung Wong W. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol*. 2001;2: RESEARCH0032.
264. Chennubhotla C, Jepson A. Half-lives of eigenflows for spectral clustering. In: Becker S, Thrun S, Obermayer K, eds. *Advances in Neural Information Processing Systems*. Cambridge, MA: MIT Press; 2003:689-696.
265. Chennubhotla C, Jepson A. Hierarchical eigensolver for transition matrices in spectral methods. In: Saul L, Weiss Y, Bottou L, eds. *Advances in Neural Information Processing Systems*. Cambridge, MA: MIT Press; 2005: 273-280.
266. Faeder JR, Blinov ML, Hlavacek WS. Rule-based modeling of biochemical systems with BioNetGen. In: Maly IV, ed. *Methods in Molecular Biology: Systems Biology*. Totowa, NJ: Humana Press; 2009.
267. Aldridge BB, Burke JM, Lauffenburger DA, et al. Physicochemical modelling of cell signalling pathways. *Nat Cell Biol*. 2006;8:1195-1203.
268. Kholodenko BN. Cell-signalling dynamics in time and space. *Nat Rev Mol Cell Biol*. 2006;7:165-176.
269. Chen WW, Niepel M, Sorger PK. Classic and contemporary approaches to modeling biochemical reactions. *Genes Dev*. 2010;24:1861-1875.
270. Jaqaman K, Danuser G. Linking data to models: data regression. *Nat Rev Mol Cell Biol*. 2006;7:813-819.
271. Chen WW, Schoeberl B, Jasper PJ, et al. Input-output behavior of ErbB signaling pathways as revealed by a mass action model trained against dynamic data. *Mol Syst Biol*. 2009;5:239.
272. Hlavacek WS. How to deal with large models? *Mol Syst Biol*. 2009;5:240.
273. Gillespie DT. Stochastic simulation of chemical kinetics. *Annu Rev Phys Chem*. 2007;58:35-55.
274. Wang RS, Saadatpour A, Albert R. Boolean modeling in systems biology: an overview of methodology and applications. *Phys Biol*. 2012;9:055001.
275. Gomez-Cabrero D, Compte A, Tegner J. Workflow for generating competing hypothesis from models with parameter uncertainty. *Interface Focus*. 2011;1:438-449.
276. Sneddon MW, Faeder JR, Emonet T. Efficient modeling, simulation and coarse-graining of biological complexity with NFsim. *Nat Methods*. 2011;8:177-183.
277. Hlavacek WS, Faeder JR, Blinov ML, et al. Rules for modeling signal-transduction systems. *Sci STKE*. 2006:re6.
278. Danos V, Feret J, Fontana W, et al. Rule-based modelling of cellular signalling. *Lect Notes Comput Sci*. 2007;4703:17-41.
279. Taniguchi Y, Choi PJ, Li GW, et al. Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science*. 2010;329:533-538.
280. Feinerman O, Veiga J, Dorfman JR, et al. Variability and robustness in T cell activation from regulated heterogeneity in protein levels. *Science*. 2008;321:1081-1084.
281. Das J, Ho M, Zikherman J, et al. Digital signaling and hysteresis characterize ras activation in lymphoid cells. *Cell*. 2009;136:337-351.
282. Feinerman O, Jentsch G, Tkach KE, et al. Single-cell quantification of IL-2 response by effector and regulatory T cells reveals critical plasticity in immune response. *Mol Syst Biol*. 2010;6:437.
283. Eldar A, Elowitz MB. Functional roles for noise in genetic circuits. *Nature*. 2010;467:167-173.
284. Neves SR. Modeling of spatially-restricted intracellular signaling. *Wiley Interdiscip Rev Syst Biol Med*. 2012;4:103-115.
285. Harris LA, Hogg JS, Faeder JR. Compartmental rule-based modeling of biochemical systems. In: *Proceedings of the 2009 Winter Simulation Conference*. Austin, TX; 2009:908-919.
286. Moraru II , Schaff JC, Slepchenko BM, et al. Virtual cell modelling and simulation software environment. *IET Syst Biol*. 2008;2:352-362.
287. Czech J, Dittrich M, Stiles JR. Rapid creation, Monte Carlo simulation, and visualization of realistic 3D cell models. *Methods Mol Biol*. 2009;500: 237-287.
288. Andrews SS, Addy NJ, Brent R, et al. Detailed simulations of cell biology with Smoldyn 2.1. *PLoS Comput Biol*. 2010;6:e1000705.
289. Takahashi K, Tanase-Nicola S, Ten Wolde PR. Spatio-temporal correlations can drastically change the response of a MAPK pathway. *Proc Natl Acad Sci U S A*. 2010;107:2473-2478.
290. Walker DC, Southgate J. The virtual cell—a candidate co-ordinator for "middle-out" modelling of biological systems. *Brief Bioinform*. 2009;10:450-461.
291. Maus C, Rybacki S, Uhrmacher AM. Rule-based multi-level modeling of cell biological systems. *BMC Syst Biol*. 2011;5:166.
292. Pitt-Francis J, Pathmanathan P, Bernabeu MO, et al. Chaste: A test-driven approach to software development for biological modelling. *Comput Phys Commun*. 2009;180:2452-2471.
293. Karr JR, Sanghvi JC, Macklin DN, et al. A whole-cell computational model predicts phenotype from genotype. *Cell*. 2012;150:389-401.
294. MacGrogan G, Jollet I, Huet S, et al. Comparison of quantitative and semiquantitative methods of assessing MIB-1 with the S-phase fraction in breast carcinoma. *Mod Pathol*. 1997;10:769-776.
295. Ali HR, Dawson SJ, Blows FM, et al. Aurora kinase A outperforms Ki67 as a prognostic marker in ER-positive breast cancer. *Br J Cancer*. 2012;106:1798-1806.
296. Reimer T, Koczan D, Muller H, et al. Tumour Fas ligand:Fas ratio greater than 1 is an independent marker of relative resistance to tamoxifen therapy in hormone receptor positive breast cancer. *Breast Cancer Res*. 2002;4:R9.
297. Bebenek M, Dus D, Kozlak J. Fas and Fas ligand as prognostic factors in human breast carcinoma. *Med Sci Monit*. 2006;12:CR457-CR461.
298. McCarthy MM, Sznol M, DiVito KA, et al. Evaluating the expression and prognostic value of TRAIL-R1 and TRAIL-R2 in breast cancer. *Clin Cancer Res*. 2005;11:5188-5194.
299. Vakkala M, Paakko P, Soini Y. Expression of caspases 3, 6 and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion. *Br J Cancer*. 1999;81:592-599.
300. Schmitz KJ, Grabellus F, Callies R, et al. Relationship and prognostic significance of phospho-(serine 166)-murine double minute 2 and Akt activation in node-negative breast cancer with regard to p53 expression. *Virchows Arch*. 2006;448:16-23.
301. Yamashita S, Masuda Y, Kurizaki T, et al. Survivin expression predicts early recurrence in early-stage breast cancer. *Anticancer Res*. 2007;27:2803-2808.
302. O'Driscoll L, Cronin D, Kennedy SM, et al. Expression and prognostic relevance of Mcl-1 in breast cancer. *Anticancer Res*. 2004;24:473-482.
303. Trere D, Montanaro L, Ceccarelli C, et al. Prognostic relevance of a novel semiquantitative classification of Bcl2 immunohistochemical expression in human infiltrating ductal carcinomas of the breast. *Ann Oncol*. 2007;18:1004-1014.
304. Thor AD, Moore II DH, Edgerton SM, et al. Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst*. 1992;84:845-855.
305. Tsutsui S, Ohno S, Murakam S, et al. Prognostic value of p53 protein expression in breast cancer; an immunohistochemical analysis of frozen sections in 514 Japanese women. *Breast Cancer*. 2001;8:194-201.
306. Xia W, Chen JS, Zhou X, et al. Phosphorylation/cytoplasmic localization of p21Cip1/WAF1 is associated with HER2/neu overexpression and provides a novel combination predictor for poor prognosis in breast cancer patients. *Clin Cancer Res*. 2004;10:3815-3824.
307. Nohara T, Ryo T, Iwamoto S, et al. Expression of cell-cycle regulator p27 is correlated to the prognosis and ER expression in breast carcinoma patients. *Oncology*. 2001;60:94-100.
308. Milde-Langosch K, Bamberger AM, Rieck G, et al. Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. *Breast Cancer Res Treat*. 2001;67:61-70.

309. Umekita Y, Ohi Y, Sagara Y, et al. Overexpression of cyclinD1 predicts for poor prognosis in estrogen receptor-negative breast cancer patients. *Int J Cancer*. 2002;98:415-418.
310. Keyomarsi K, Tucker SL, Buchholz TA, et al. Cyclin E and survival in patients with breast cancer. *N Engl J Med*. 2002;347:1566-1575.
311. Harigopal M, Berger AJ, Camp RL, et al. Automated quantitative analysis of E-cadherin expression in lymph node metastases is predictive of survival in invasive ductal breast cancer. *Clin Cancer Res*. 2005;11:4083-4089.
312. Dolled-Filhart M, McCabe A, Giltnane J, et al. Quantitative in situ analysis of beta-catenin expression in breast cancer shows decreased expression is associated with poor outcome. *Cancer Res*. 2006;66:5487-5494.
313. Morris SF, O'Hanlon DM, McLaughlin R, et al. The prognostic significance of CD44s and CD44v6 expression in stage two breast carcinoma: an immunohistochemical study. *Eur J Surg Oncol*. 2001;27:527-531.
314. Bircan S, Kapucuoglu N, Baspinar S, et al. CD24 expression in ductal carcinoma in situ and invasive ductal carcinoma of breast: an immunohistochemistry-based pilot study. *Pathol Res Pract*. 2006;202:569-576.
315. Morohashi S, Kusumi T, Sato F, et al. Decreased expression of claudin-1 correlates with recurrence status in breast cancer. *Int J Mol Med*. 2007;20:139-143.
316. Salvucci O, Bouchard A, Baccarelli A, et al. The role of CXCR4 receptor expression in breast cancer: a large tissue microarray study. *Breast Cancer Res Treat*. 2006;97:275-283.
317. Gilcrease MZ, Kilpatrick SK, Woodward WA, et al. Coexpression of alpha-6beta4 integrin and guanine nucleotide exchange factor Net1 identifies node-positive breast cancer patients at high risk for distant metastasis. *Cancer Epidemiol Biomarkers Prev*. 2009;18:80-86.
318. Kamat AA, Fletcher M, Gruman LM, et al. The clinical relevance of stromal matrix metalloproteinase expression in ovarian cancer. *Clin Cancer Res*. 2006;12:1707-1714.
319. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Flt-4-positive vessel density correlates with vascular endothelial growth factor-d expression, nodal status, and prognosis in breast cancer. *Clin Cancer Res*. 2003;9:5313-5317.
320. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Prognostic significance of vascular endothelial growth factor D in breast carcinoma with long-term follow-up. *Clin Cancer Res*. 2003;9:716-721.
321. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Clinicopathological significance of vascular endothelial growth factor-C in breast carcinoma with long-term follow-up. *Mod Pathol*. 2003;16:309-314.
322. Chen HH, Su WC, Lin PW, et al. Hypoxia-inducible factor-1alpha correlates with MET and metastasis in node-negative breast cancer. *Breast Cancer Res Treat*. 2007;103:167-175.
323. Cooke VG, LeBleu VS, Keskin D, et al. Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer Cell*. 2012;21:66-81.
324. Schule J, Bergkvist L, Hakansson L, et al. Down-regulation of the CD3-zeta chain in sentinel node biopsies from breast cancer patients. *Breast Cancer Res Treat*. 2002;74:33-40.
325. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia*. 2006;8:190-198.
326. Tsutsui S, Yasuda K, Suzuki K, et al. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep*. 2005;14:425-431.
327. Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. *Cytokine Growth Factor Rev*. 2006;17:325-337.
328. Zerkowski MP, Camp RL, Burtness BA, et al. Quantitative analysis of breast cancer tissue microarrays shows high cox-2 expression is associated with poor outcome. *Cancer Invest*. 2007;25:19-26.
329. Kluger HM, Dolled-Filhart M, Rodov S, et al. Macrophage colony-stimulating factor-1 receptor expression is associated with poor outcome in breast cancer by large cohort tissue microarray analysis. *Clin Cancer Res*. 2004;10:173-177.
330. Henry LR, Lee HO, Lee JS, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. *Clin Cancer Res*. 2007;13:1736-1741.
331. Paulsson J, Sjoblom T, Micke P, et al. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. *Am J Pathol*. 2009;175:334-341.
332. Barroso MM. Quantum dots in cell biology. *J Histochem Cytochem*. 2011;59:237-251.
333. van der Krogt GNM, Ogink J, Ponsioen B, et al. A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. *PLoS One*. 2008;3:e1916.
334. Day RN, Davidson MW. Fluorescent proteins for FRET microscopy: monitoring protein interactions in living cells. *BioEssays*. 2012;34:341-350.
335. McNamara G, Gupta A, Reynaert J, et al. Spectral imaging microscopy web sites and data. *Cytometry A*. 2006;69:863-871.
336. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol*. 2010;28:1248-1250.
337. Chatr-aryamontri A, Breitkreutz B-J, Heinicke S, et al. The BioGRID interaction database: 2013 update. *Nucl Acids Res*. 2013;41:D816-D823.
338. Fernçndez-Suçrez XM, Galperin MY. The 2013 Nucleic Acids Research Database Issue and the online Molecular Biology Database Collection. *Nucl Acids Res*. 2013;41:D1-D7.
339. Orloff DN, Iwasa JH, Martone ME, et al. The cell: an image library-CCDB: a curated repository of microscopy data. *Nucl Acids Res*. 2013;41:D1241-D1250.
340. Krupp M, Itzel T, Maass T, et al. CellLineNavigator: a workbench for cancer cell line analysis. *Nucl Acids Res*. 2013;41:D942-D948.
341. Conlon S, Colgan A, Allen D, et al. Comparison of dual-colour chromogenic in-situ hybridization and fluorescence in-situ hybridization in the assessment of HER2 gene amplification in breast carcinoma. *Histopathology*. 2011;58:319-321.
342. Arnould L, Denoux Y, MacGrogan G, et al. Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. *Br J Cancer*. 2003;88:1587-1591.
343. Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. *Hum Pathol*. 2007;38:1105-1122.
344. Gupta D, Middleton LP, Whitaker MJ, et al. Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. *Am J Clin Pathol*. 2003;119:381-387.
345. Van Laere SJ, Van der Auwera I, Van den Eynden GG, et al. Nuclear factor-kappaB signature of inflammatory breast cancer by cDNA microarray validated by quantitative real-time reverse transcription-PCR, immunohistochemistry, and nuclear factor-kappaB DNA-binding. *Clin Cancer Res*. 2006;12:3249-3256.
346. Biswas DK, Shi Q, Baily S, et al. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci U S A*. 2004;101:10137-10142.
347. Kielhorn E, Provost E, Olsen D, et al. Tissue microarray-based analysis shows phospho-beta-catenin expression in malignant melanoma is associated with poor outcome. *Int J Cancer*. 2003;103:652-656.
348. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
349. Verghese ET, Shenoy H, Cookson VJ, et al. Epithelial-mesenchymal interactions in breast cancer: evidence for a role of nuclear localized beta-catenin in carcinoma-associated fibroblasts. *Histopathology*. 2011;59:609-618.
350. Widschwendter A, Tonko-Geymayer S, Welte T, et al. Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. *Clin Cancer Res*. 2002;8:3065-3074.
351. Nevalainen MT, Xie J, Torhorst J, et al. Signal transducer and activator of transcription-5 activation and breast cancer prognosis. *J Clin Oncol*. 2004;22:2053-2060.
352. Orend G, Hunter T, Ruoslahti E. Cytoplasmic displacement of cyclin E-cdk2 inhibitors p21Cip1 and p27Kip1 in anchorage-independent cells. *Oncogene*. 1998;16:2575-2583.
353. Asada M, Yamada T, Ichijo H, et al. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *EMBO J*. 1999;18:1223-1234.
354. Zhou BP, Liao Y, Xia W, et al. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol*. 2001;3:245-252.
355. Huang LE, Arany Z, Livingston DM, et al. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem*. 1996;271:32253-32259.
356. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*. 1999;59:5830-5835.
357. Habashy HO, Rakha EA, Aleskandarany M, et al. FOXO3a nuclear localisation is associated with good prognosis in luminal-like breast cancer. *Breast Cancer Res Treat*. 2011;129:11-21.

Eric S. Schafer, Judith Margolin, David G. Poplack, and Karen R. Rabin

26

Molecular Genetics of Acute Lymphoblastic Leukemia

Introduction

Acute lymphoblastic leukemia (ALL) is a sharply contrasting disease in the pediatric and adult populations. In children, it is both the most common leukemia and the most common malignancy. Childhood ALL has been emblematic of medical progress, with steady improvement over the past 50 years and a current 5-year event-free survival (EFS) rate of over 85%[1] (Figure 26-1). In contrast, in adults, ALL constitutes a minority of the leukemias and a tiny fraction of all malignancies. In addition, the long-term disease-free survival for adults with ALL is poor, in the range of 30% to 40%.[2] An understanding of molecular genetics is playing an increasingly important role in optimizing therapy in pediatric ALL, defining distinct prognostic subgroups for which therapy can be tailored so that low-risk patients are spared unnecessary toxicity, while high-risk patients receive the intensive therapy most likely to effect a cure. Furthermore, some of the discrepancy between cure rates in childhood and adult ALL can be explained by noting that positive prognostic genetic lesions tend to be more common in children, whereas genetic lesions that are associated with more resistant disease tend to be more prevalent in older patients. New insights into the molecular biology of ALL may both increase the ability to more accurately risk stratify patients and identify targets for novel therapeutics that could increase survival and decrease toxicity in all patients with ALL.

Prognostic Factors

The cornerstone of ALL therapy is stratification of patients into different risk groups based on a combination of clinical, laboratory, and molecular features, so that the type and intensity of therapy may be tailored appropriately.[1] For example, in children, three major factors are used to assign risk-based therapy at diagnosis. First is the Rome-National Cancer Institute (NCI) risk status, which defines high-risk ALL as those with an age less than 1 year or more than 9.99 years, and/or initial white blood cell (WBC) count greater than 50,000/μL. In adults, older age and higher WBC are associated with increasing risk. Second is early response to treatment. Third, and increasingly important, is molecular genetic alterations of the tumor cells.

Overview of Molecular Genetics of ALL

Cytogenetic analysis using karyotypic characterization and fluorescence in situ hybridization (FISH) is a crucial element in diagnostic evaluation. Translocations are relatively common in ALL and generally cause two types of events: a proto-oncogene may be brought into the proximity of a T-cell receptor or immunoglobulin locus, causing its overexpression; or the genes at the breakpoints of the rearranged chromosomes, often transcription factors, may fuse to form a new, chimeric protein that is oncogenic because of altered properties and/or expression patterns.[3]

Although ALL has traditionally been defined by recurrent karyotypic changes, about one quarter of patients lack characteristic chromosomal rearrangements. In addition, none of the known rearrangements has been shown to be both necessary and sufficient for leukemogenic transformation. Recent technological advances in high-resolution genomic sequencing coupled with active large-scale support by initiatives such as the NCI TARGET (Therapeutically Applicable Research to Generate Effective Treatments; www.target.gov) project have led to large sample surveys identifying more than 30 validated recurring tumor-specific somatic subchromosomal mutations in a majority of patients with ALL. Among these, most occur in key signaling pathways such as B-cell development/differentiation, the TP53/RB

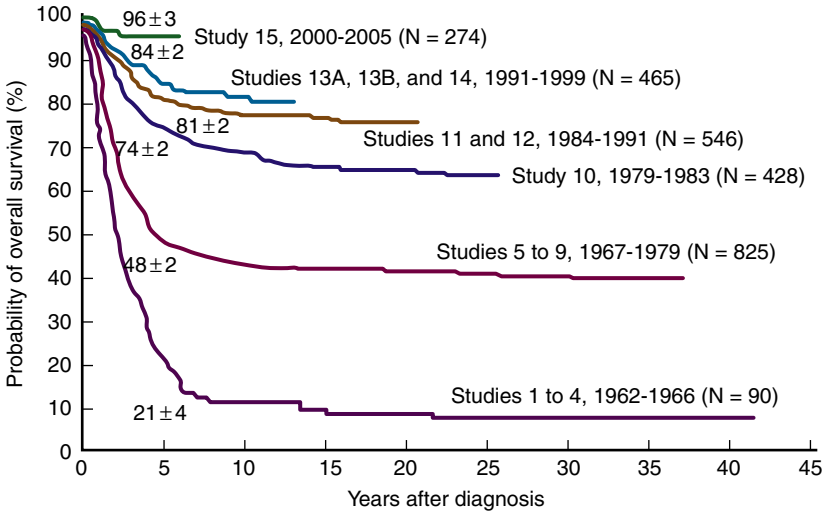


Figure 26-1 Kaplan-Meier analysis of overall survival in 2628 children with newly diagnosed acute lymphocytic leukemia (ALL). The patients participated in 15 consecutive studies conducted at St. Jude Children's Research Hospital from 1962 to 2005. The 5-year overall survival estimates (± SE) are shown, except for Study 15, for which preliminary results at 4 years are provided. *(From Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia.* N Engl J Med. *2006;354:166-178, with permission.)*

tumor suppressor pathway, and Ras and Janus kinase signaling.[4] These advances, along with microarray-based analyses of gene expression and epigenetic profiles, have elucidated important basic science aspects of leukemogenesis (Table 26-1). In addition, some of these findings have already translated into several promising novel therapies.

Abnormalities of Chromosome Number (Ploidy)

Ploidy can be assessed either by chromosome number or by flow cytometry using the DNA index (DI), the ratio of fluorescence in leukemic blasts compared to normal cells. Normal diploid cells have 46 chromosomes and a DI of 1.0, hyperdiploid cells have higher values, and hypodiploid cells lower. Hyperdiploidy is further classified as "low" and "high" (greater than 50 chromosomes).

Hypodiploid cases constitute approximately 6% of pediatric and 2% to 8% of adult ALL.[5] Those with fewer than 45 chromosomes have significantly worse outcome, with the worst outcome occurring in near-haploid cases (24 to 28 chromosomes).[5] The adverse prognostic impact in adults is somewhat weaker. "Pseudodiploid" cases, with normal chromosome number but structural abnormalities, also do relatively poorly. Rare cases with near triploidy or near tetraploidy (more than 80 chromosomes) have traditionally been associated with poor outcome. However, more recent reports analyzed from studies using modern therapies refute this claim, showing that these lesions should be classified prognostically as neutral[6] or even favorable.[7]

Hyperdiploidy occurs in about 35% of pediatric and 25% of adult ALL cases.[8] In children, ALL with more than 50 chromosomes, or simply the simultaneous trisomies of 4 and 10 (and less importantly 17), is an independent positive prognostic indicator. In adults, the prognostic implications are less clear. Although the biologic basis of hyperdiploidy is poorly defined, it often co-occurs with other favorable risk factors as well as *Ras* and *FLT3* mutations and *FHIT* hypermethylation.

Genetic Abnormalities in ALL

B-ALL

Abnormalities of Chromosome Structure

(*ETV6-RUNX1*), t(12;21)(p13;q22)

The *ETV6-RUNX1* (*TEL-AML1*) fusion protein formed by the t(12;21)(p13;q22) translocation is the most frequent abnormality in children (25%), whereas it is much rarer in adults (2%). *ETV6* (ETS variant gene 6) is also known as *TEL* (translocation-ETS-leukemia). *RUNX1* (runt-related transcription factor 1) is also known as *AML1* or *CBFA2* (core binding factor A2). In nearly all cases the translocation is cryptic, involving a region too small to be detected by karyotype.

ETV6 encodes a widely expressed nuclear protein belonging to the Ets family of transcription factors, which are involved in diverse developmental processes including the establishment of embryonic and adult hematopoiesis. A helix-loop-helix (HLH) region known as the ETS domain allows DNA binding for transcriptional regulation, and an HLH region known as the pointed domain appears to facilitate self-association (Figure 26-2, *A*). *RUNX1* is a transcription factor with highly restricted expression in hematopoietic cells and developing ganglions but that is required for transcription of several hematopoietic-specific

Table 26-1 Selected Genetic Abnormalities Associated with ALL

Chromosomal Abnormality	Genes Involved	Pediatric (%)	Adult (%)	Mechanism of Transformation	Prognostic Impact
Pre-B ALL					
t(12;21)(p13;q22)	*ETV6-RUNX1*	25	2	Represses AML1 function as transcriptional activator	Favorable
t(1;19)(q23;p13)	*E2A-PBX1*	6	3	Promotes PBX1 function as transcriptional activator	Formerly poor; negated by intensive therapy
t(17;19)(q23;p13)	*E2A-HLF*	<1	<1	Repression of E2A and antiapoptotic effects (?)	Poor
t(9;22)(q34;q11)	*BCR-ABL*	3	25	Increased tyrosine kinase activity	Poor; partially ameliorated by imatinib (Gleevec)
t(4;11)(q21;q23)	*MLL-AF4*	8	10	Disruption of Hox expression patterns	Poor
iAMP21	*RUNX1*, mir-802, and DSCR	2		Initiates development of secondary lesions (in, e.g., *IKZF1*, *ETV6*, *RB1*)	Poor
PAX5 mutation	*PAX5*	30	12	Disrupts normal B-cell maturation process	No known significance
EBF1 mutation	*EBF1*	4	1.5	Disrupts normal B-cell development/decreases PAX5 expression	No known significance
IKZF1 mutation	Ikaros	80 (Ph^+) 7 (Ph^-)	63 (Ph^+) 19 (Ph^-)	Activation of JAK/STAT and increased expression of Bcl-xL	Poor
CRLF2 overexpression	*CRLF2*	8 (15% in high risk and 50% of DS-ALL)	10-15	Upregulation of JAK/STAT and PI3K/mTOR pathways	No significance in DS; controversial in SR, poor in HR
JAK mutation	*JAK1*, *JAK2*, *JAK3*	10 in B cell (mostly JAK2)	17 in T cell (mostly JAK1)	Constitutive activation of the JAK tyrosine kinase	Poor
CREBBP mutation (relapsed ALL)	*CREBBP*	19	N/A	Impaired transcriptional regulation of CREBBP targets	No significance
T ALL					
NOTCH mutation	*NOTCH1*	>50	>50	Constitutively activated NOTCH1 causing activation of downstream targets (e.g., c-Myc, cyclin D, and NFκB)	Improved
t(1;14)(p34;q11)	*TAL1*, *TCRα/δ*	7	12	Repression of E2A transcriptional activity	Poor vs. no prognostic significance
t(11;14)(p15;q11)	*LMO1*, *TCRα/δ*	<1	<1	LMO1 activation; repression of E2A transcriptional activity	Unknown
t(11;14)(p13;q11)	*LMO2*, *TCRα/δ*	1	<1	LMO2 activation; repression of E2A transcriptional activity	Unknown
t(10;14)(q24;q11); t(7;10)(q35;q24)	*HOX11*, *TCRδ*, or *TCRβ*	0.7	8	Dysregulated expression of intact HOX11	Favorable if intensive therapy
t(5;14)(q35;q32); t(5;14)(q35;q11)	*HOX11L2*, *BCL11B*, or *TCRδ*	2.5	1	HOX11L2 activation	Poor vs. no prognostic significance
t(8;14)(q24;q11)	*MYC*, *TCR*	<1	<1	MYC overexpression	Unknown
t(7;19)(q35;p13)	*LYL1*, *TCRβ*	1.5	2.5		Unknown
Mature B ALL					
t(8;14)(q24;q32)	*MYC*, *IgH*	2	4	MYC overexpression	
t(8;22)(q24;q11)	*MYC*, *Igλ*	<1	<1		
t(2;8)(p12;q24)	*Igκ*, *MYC*	<1	<1		

DS, Down syndrome; HR, high risk; SR, standard risk.

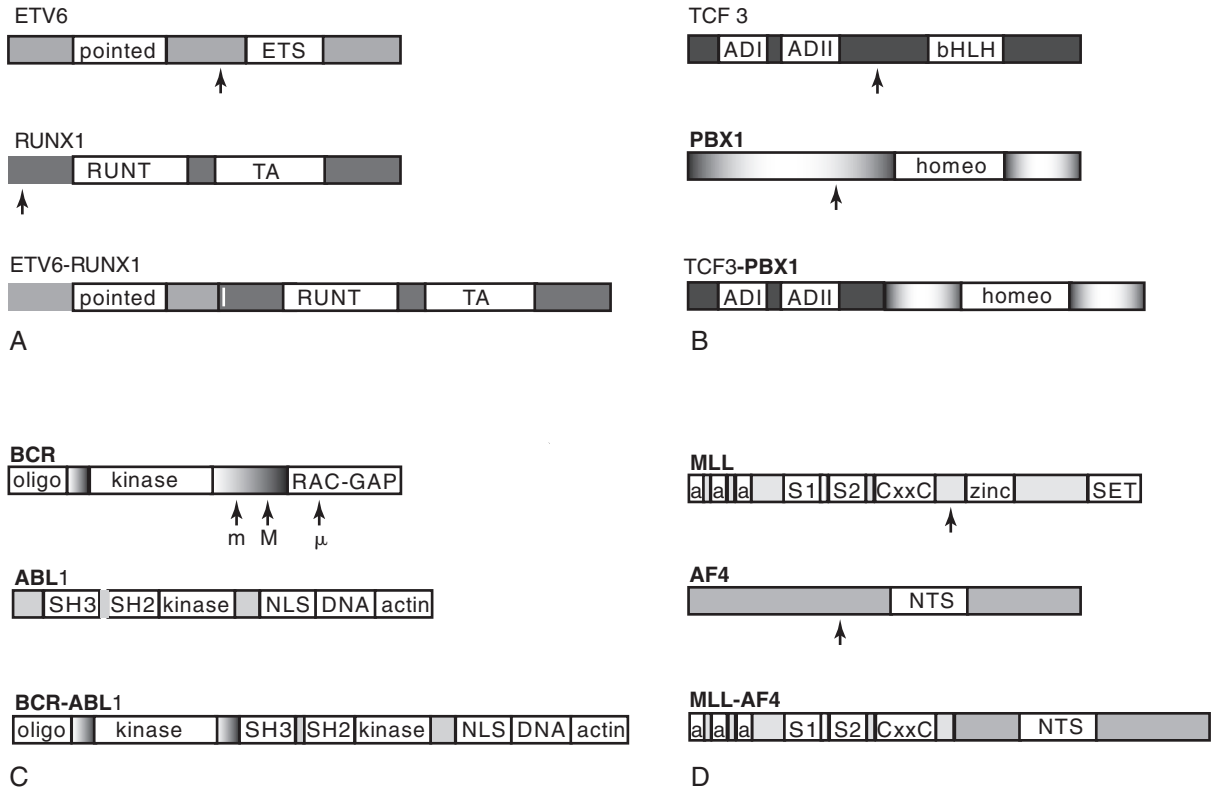


Figure 26-2 Schematic of key domains of the genes involved in several principal translocations in acute lymphocytic leukemia (ALL) and the translocation products. *Arrows* indicate common breakpoints. Note that loss of the domain conferring sequence specificity to transcription factor binding occurs in ETV6-RUNX1 (ETS domain of TEL) and TCF3-PBX1 (bHLH domain of E2A). Gene regions are not drawn to scale. **(A)** ETV6-RUNX1. *TA,* Transactivation domain. **(B)** TCF3-PBX1. *ADI* and *ADII,* Activation domains I and II; *bHLH,* basic helix-loop-helix, sequence-specific DNA-binding domain; *homeo,* homeobox domain. **(C)** BCR-ABL. All three breakpoint regions are indicated (*M,* major, for p210 protein; *m,* minor, for P190 protein; μ, associated with P230 protein). Only the P190 fusion product is illustrated. *oligo,* Oligomerization domain; *kinase,* serine-threonine kinase domain; *RAC-GAP,* RAS-like GTPase; *SH3, SH2,* and *kinase,* SRC homology domains; *kinase,* tyrosine kinase domain; *NLS,* nuclear localization domains; *DNA,* DNA-binding site; *actin,* G and F actin binding site. **(D)** Mixed lineage leukemia. *a,* AT-hook; *CxxC,* cysteine-rich motif homologous to DNA methyltransferase; *S1* and *S2,* subnuclear localization domains; *NTS,* nuclear targeting sequence; *zinc,* zinc fingers.

genes, and may organize the factor complex necessary for lineage-specific transcription.

The *ETV6-RUNX1* fusion protein is widely expressed because of the *ETV6* promoter and converts *RUNX1* from a transcriptional activator to a repressor. The exact mechanism of repression is unclear, as is the manner in which this mediates leukemogenesis. *ETV6-RUNX1* overexpression causes leukemia in only a minority of mouse models, with low penetrance and prolonged latency, suggesting that additional events are crucial for full transformation. A frequent secondary event in *ETV6-RUNX1*+ ALL is loss of heterozygosity (LOH), deletion, or otherwise downregulated expression of the remaining normal copy of *ETV6*, suggesting a potential role for *ETV6* as a tumor suppressor.

ETV6-RUNX1 positivity tends to occur in children 1 to 10 years of age, and nearly exclusively in $CD10^+$ B-precursor ALL. In the past, cases characterized by *ETV6-RUNX1*$^+$ ALL had a hallmark tendency to relapse late, with excellent chemosensitivity and salvage rate. On modern treatment regimens *ETV6-RUNX1* relapsed disease is extremely rare and, although survival for all subtypes of ALL in children with contemporary chemotherapy strategies has improved, *ETV6-RUNX1* has retained positive prognostic significance.[9] When relapse does occur, evidence suggests that it may represent evolution of a new leukemic clone from the preleukemic *ETV6-RUNX1*$^+$ cell of origin.

TCF3-PBX1, t(1;19)(q23;p13)

The *TCF3-PBX1* fusion protein, associated with the t(1;19)(q23;p13) translocation, is the second most common translocation in pediatric ALL, occurring in approximately 6% of all pre-B ALL.[10] It is a rare (3%) and adverse feature in adults. The fusion protein combines the two activation domains of the basic helix-loop-helix (bHLH) transcription factor *TCF3* (previously *E2A*) on chromosome 19 with the homeobox (*HOX*) gene *PBX1* (for pre-B cell homeobox 1) on chromosome 1, resulting in a strong transcriptional activator effect on PBX1 (see Figure 26-2, *B*). *TCF3* is a transcriptional activator critical in lymphocyte development, as well as widely expressed and influential in diverse cellular

processes. *PBX1* belongs to the TALE (three amino acid loop extension) class of atypical homeodomain proteins. The homeodomain mediates both DNA-binding and *HOX* gene interaction.

The *TCF3-PBX1* chimeric transcription factor strongly activates a subset of *HOX* genes normally regulated by *PBX1*. The basis for its transforming ability may be reduction of wild-type *TCF3* levels; aberrant activation of *PBX1* targets in pre-B cells; or activation of targets not normally regulated by *PBX1* that are affected by the *TCF3-PBX1* fusion protein.[11] Fusion protein overexpression in mouse models causes a variety of leukemias, although not B-lineage ALL, suggesting potent non–lineage-specific transforming activity. Unlike other ALL translocations, t(1;19)$^+$ ALL does not show evidence of in utero origin.

TCF3-PBX1 positivity often coincides with other high-risk factors. Early studies indicated an independent adverse prognostic impact, but on modern intensive pediatric regimens, survival is equivalent.[10] The t(1;19) occurs most often as an unbalanced translocation. Cases with a balanced translocation do more poorly in some studies but not others.

TCF3-HLF, t(17;19)(q23;p13)

The t(17;19) translocation is a much rarer event, occurring in approximately 1% of pediatric ALL and rarely in adults.[12] The *HLF* (for hepatic leukemia factor) fusion partner is a transcription factor not normally expressed in hematopoietic cells. Potential oncogenic effects of the fusion protein include repression of normal *TCF3* function, altered transcriptional activity of *HLF*, and promotion of lymphoblast survival, possibly as the result of antiapoptotic effects. *TCF3-HLF* ALL tends to occur in adolescents and is frequently associated with hypercalcemia, disseminated intravascular coagulopathy (DIC), a cIgM-negative, low CD10 positivity pro-B cell immunophenotype, and a poor prognosis despite intensive chemotherapy.

BCR-ABL1, t(9;22)(q34;q11)

The t(9;22) translocation was the first recurrent chromosomal abnormality identified in human cancer, in association with chronic myelocytic leukemia (CML). This translocation, known as the Philadelphia chromosome (Ph), is necessary and sufficient in transformation to the preleukemic, myeloproliferative neoplasm CML. *BCR-ABL1* is also the most common translocation noted in adult ALL, with a prevalence of 25%, where it is known to be leukemogenic but not in itself sufficient to cause disease. The occurrence of the Ph in ALL increases with age and is therefore seen only in about 3% of childhood ALL cases.[13] Philadelphia-positive ALL is primarily a CD10$^+$ precursor B ALL with frequent coexpression of myeloid markers. Patients with Ph$^+$ ALL tend to be older, present with higher leukocyte and peripheral blast counts, have central nervous system (CNS) involvement, and historically have exhibited lower induction remission rates, shorter remission durations, and very poor overall survival (OS). Monosomy 7 and/or loss of 9p are secondary aberrations that may be associated with worse outcomes.

The Philadelphia chromosome is formed by in-frame fusion of the 5′ portion of *BCR* (for breakpoint cluster region) on chromosome 22 to the 3′ portion of the tyrosine kinase *C-ABL1* on chromosome 9, a proto-oncogene that is part of the *RAS* signaling pathway (see Figure 26-2, *C*). The fusion protein upregulates *ABL1* tyrosine kinase activity. Two main fusion proteins occur, which differ in *BCR* breakpoint. Breaks within the 5.8-kb major breakpoint cluster region (M-BCR), occurring in CML and 25% of adult Ph$^+$ ALL, form a 210-kDa protein known as p210. In the remainder of adult ALL and the majority of pediatric ALL, the breakpoint occurs further upstream, in the minor breakpoint cluster region (m-BCR), forming a 185- to 190-kDa protein usually known as p190. An additional breakpoint generates a 230-kDa protein associated with a rare CML variant with neutrophilia and occasionally with classic CML. All three transcripts can be detected at very low levels using sensitive PCR techniques. It has been suggested that the p190 protein arises de novo, whereas the p210 protein may represent the blast crisis of a previously unrecognized CML. Other features that may distinguish cases that originated as CML include basophilia, marked splenomegaly, and persistence of the *BCR-ABL1* fusion protein in hematopoietic precursor cells of all lineages following remission.

Treatment of CML and Ph$^+$ ALL was revolutionized by the development of imatinib mesylate, also known as STI-571 or Gleevec, introduced in 2001.[14] Imatinib, a selective tyrosine kinase inhibitor, was the first molecularly targeted therapy to attain large-scale clinical success, fulfilling the goals of antitumor selectivity and low systemic toxicity. Despite its success, however, it has not been effective as a single agent because of the rapid development of resistance. Nevertheless, in ALL, when combined with standard cytotoxic chemotherapy, imatinib has dramatically improved patient outcomes. In adults, imatinib plus chemotherapy alone or as a bridge to transplant has improved 4-year OS from roughly 15% to between 38% and 54%.[15] In children, outcomes with the addition of imatinib have been even more striking. Children's Oncology Group (COG) study AALL0031 demonstrated that addition of imatinib to high-intensity chemotherapy improved 3-year EFS to 80% compared to historical controls of 35%.[13] Outcomes in patients treated with chemotherapy plus imatinib were similar to those in patients receiving sibling donor bone marrow transplant (BMT), suggesting that BMT in first remission may no longer be the treatment of choice for Ph$^+$ ALL

in children.[13] New developments in the treatment of Ph+ ALL include more potent second-generation tyrosine kinase inhibitors; dual SRC and *BCR-ABL1* kinase inhibitors; and combination therapy with a farnesyl transferase or a PI3 kinase inhibitor.

MLL, 11q23 Rearrangements

MLL gene rearrangements (*MLL*-r) occur in 8% of pediatric ALL and 10% of adult ALL and constitute the most frequent abnormality in infant ALL, occurring in 60% to 70% of cases.[16] *MLL*-r is also associated with AML, particularly secondary malignancies following anthracyclines and epipodophyllotoxins. *MLL*-r leukemias are unusual in two respects: the N terminus of *MLL* forms a fusion protein with the C termini of more than 70 different partners, including itself;[17] and *MLL*-r are found in both ALL and AML, whereas most other translocations are lineage specific. Indeed, *MLL* takes its name, "mixed lineage leukemia," from this distinguishing feature (it is also known as *HRX* or *HTRX* for homology to *trithorax* in *Drosophila*, and *ALL1* for involvement in ALL). *MLL*-r ALL has a unique gene expression pattern suggestive of arrest at an earlier hematopoietic progenitor stage. The *MLL-AF4* fusion protein formed by t(4;11) is the most common *MLL* translocation in ALL, making up 70% of cases. The *MLL-ENL* fusion formed by t(11;19) comprises another 13%.

Infant ALL with *MLL*-r tends to be associated with age less than 6 months, massive tumor burden, organomegaly, frequent CNS involvement, coexpression of myeloid antigens, and CD10-negative pro-B immunophenotype. *MLL*-r is a very poor prognostic feature in infant ALL, and a poor prognostic feature in children more than 1 year of age. One surprising exception is *MLL-ENL*, associated with good prognosis in both T ALL and patients age 1 to 9 years.[18]

MLL is a large protein consisting of multiple motifs, including AT-hook DNA-binding domains, transcriptional activation and repression zinc finger domains, and a highly conserved SET domain that regulates homeotic (*Hox*) promoters. Several motifs are homologous to the *Drosophila* trithorax protein, which maintains appropriate expression of the *Hox* genes controlling segment determination. In normal hematopoiesis, MLL is required to generate stem-cell progenitors and to establish both lymphoid and myeloid lineages. In general, the many *MLL* fusion partners fall in the broad categories of signaling molecules and nuclear transcription factors.

Because *Hox* genes can induce leukemia, it is thought that the MLL-r protein mediates leukemogenesis through disruption of normal *Hox* expression patterns. The contributions of *MLL*'s many fusion partners to leukemic transformation remain unclear, as they do not all share structural or functional similarities (see Figure 26-2, *D*). One common function may be converting MLL to a constitutive transcriptional effector. In addition, there is a growing body of evidence that *MLL*-r leukemias are driven primarily by epigenetic dysregulation, including aberrations in DNA and histone methylation and histone acetylation.[19]

Both infant and treatment-related leukemias show nonrandom clustering of *MLL* breakpoints within exons distinct from the breakpoints in de novo *MLL*-r leukemias, suggesting a shared mechanism of leukemogenesis. One hypothesis is that infant ALL arises from in utero exposure to topoisomerase II inhibitors, analogous to the initiating insult in therapy-related *MLL*-r leukemias. Epidemiologic studies of the association between infant leukemia and in utero exposure to topoisomerase II inhibitors have identified a modest association between dietary intake and *MLL*-r AML, and a stronger association between certain medications and insecticides and *MLL*-r ALL.[20]

Event-free survival in infant ALL is generally poor, ranging from 20% to 40%, with t(4;11) having a particularly dismal prognosis. Induction rates are generally comparable to other types of ALL, but early relapse is frequent, usually within a year of diagnosis. *MLL*-r ALL shows relative resistance in vitro to glucocorticoids and L-asparaginase, and sensitivity to cladribine and Ara-C. High-dose Ara-C has been incorporated into infant ALL regimens with modest success. The receptor tyrosine kinase FLT3 is highly expressed in *MLL*-r ALL[21] and the use of *FLT3* inhibitors and other novel molecularly targeted therapies is under investigation.

Intrachromosomal Amplification of Chromosome 21 (iAMP21)

Recently, a novel recurrent chromosomal abnormality, the intrachromosomal amplification of chromosome 21 (iAMP21), has been identified in a rare (1.5% to 2% of ALL) and very poor prognostic subgroup of patients with ALL. In childhood ALL, iAMP21 patients tend to be older, with lower initial white blood cell and platelet counts. Clinical outcomes are poor, with relapse rates ranging from 38% to 61% depending on the treatment regimen.[22] Most known abnormalities take place in a 5.1-Mb common region of amplification (CRA) on the long arm of chromosome 21 that includes *RUNX1*, miR-802, and genes mapping to the Down syndrome critical region (DSCR). ALL with iAMP21 consistently has multiple copies of *RUNX1* identified in array-based comparative genomic hybridization and FISH assays (Figure 26-3, *A*). However, gene expression assays fail to show either overexpression of *RUNX1* RNA or a unique iAMP21 signature as compared to other ALL samples. Although the basis for the increased risk of relapse in this subgroup remains unclear, iAMP21 is now used to stratify patients to receive intensified chemotherapy on several current ALL treatment protocols.[23]

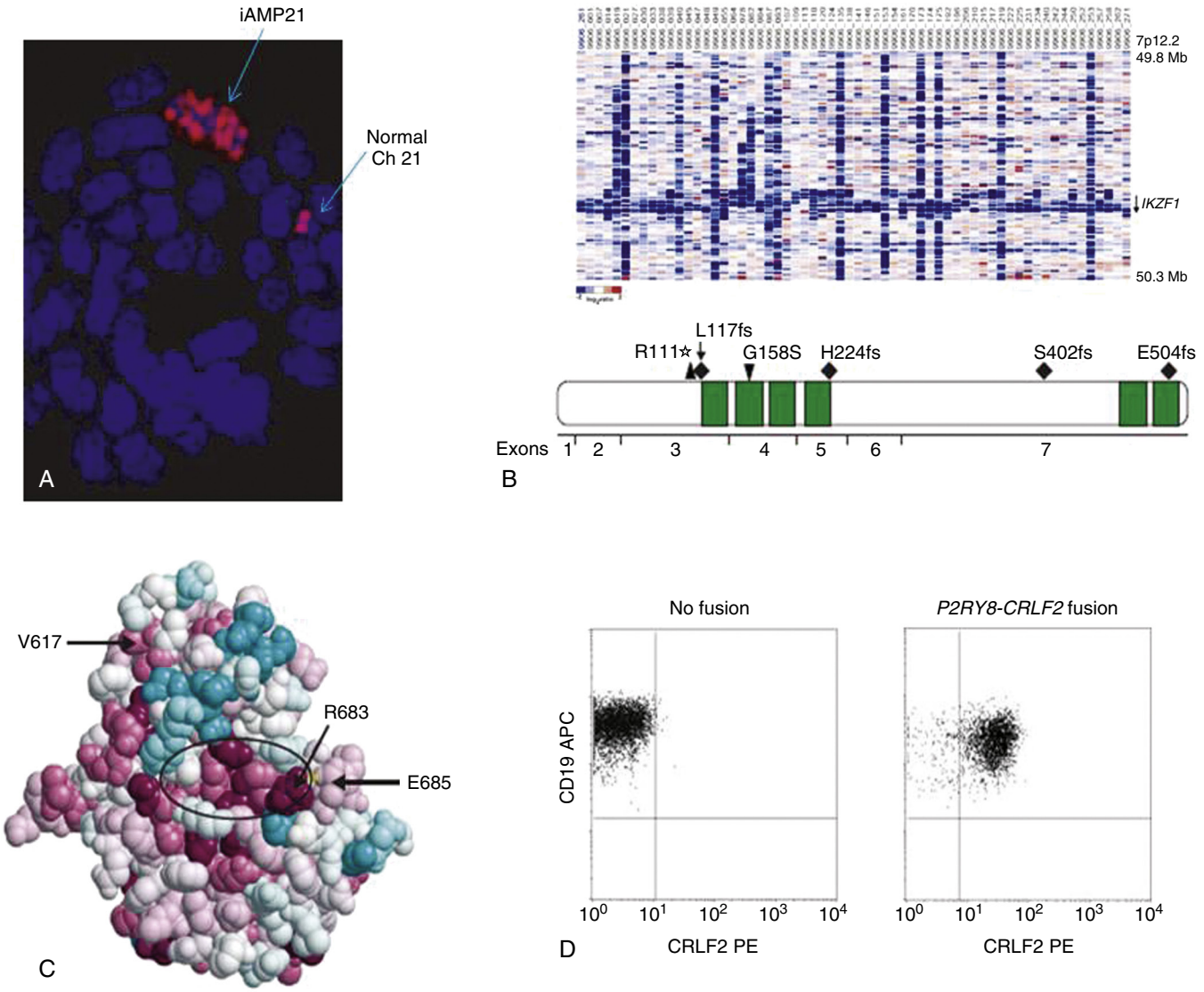


Figure 26-3 Recently identified chromosomal aberrations in ALL **(A)** iAMP21: Metaphase FISH demonstrates multiple RUNX1 signals (*red*) on the chromosome 21 with the iAMP21 aberration (*top*), whereas a single signal is noted on each normal chromosome 21 *(right)*. *(Modified from Harrison CJ. Cytogenetics of pediatric and adolescent acute lymphoblastic leukemia.* Br J Haematol. *2009;144:147-156, with permission.)* **(B)** IKZF1 deletions and mutations: DNA copy number heatmap *(top)* demonstrating deletions *(blue)* at the IKZF1 locus in a cohort of ALL cases, and primary protein structure of IKAROS *(bottom)* showing the location of the six zinc fingers *(green)* and missense *(downward-pointing arrowhead)*, frameshift *(diamonds)*, and nonsense *(upward-pointing arrowhead)* mutations observed in six ALL cases. *(From Mullighan C, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia.* N Engl J Med. *2009;360:470-480, with permission.)* **(C)** JAK2 mutations: Amino acid structural model showing that R683, the amino acid most frequently affected by point mutations in ALL, is situated on a rim of highly conserved *(red)*, exposed amino acids that make up an apparent binding pocket (encircled by *ellipse*) that is distinct from the V617 amino acid commonly mutated in myeloproliferative neoplasms. *(From Bercovich D, et al. Mutations of JAK2 in acute lymphoblastic leukemias associated with Down's syndrome.* Lancet. *2008;372:1484-1492, with permission.)* **(D)** CRLF2 overexpression: Flow cytometric analysis of cell surface expression of CRLF2 demonstrates CRLF2 overexpression in ALL cases with the *P2RY8-CRLF2* fusion and not in fusion-negative cases; CD19 co-staining was performed to demonstrate selectivity for the leukemic cell population. *(Modified from Mullighan C, et al. Rearrangement of CRLF2 in B-progenitor and Down syndrome associated acute lymphoblastic leukemia.* Nat Genet. *2009;41:1243-1246, with permission.)*

Submicroscopic Abnormalities

PAX5 and *EBF1*

In a landmark study, Mullighan and colleagues performed genome-wide analysis of 242 pediatric ALL patient samples using high-resolution single-nucleotide polymorphism arrays and genomic DNA sequencing. They discovered that precursor B-cell ALL samples on average carried 6.63 copy number alterations, indicating relative genomic stability, but confirming the presence of additional subchromosomal cooperating lesions in ALL cases with known leukemogenic karyotypic abnormalities. Notably, about 40% of cases carried aberrations in genes that regulate B lymphocyte development, including *EBF1* and *PAX5*.[24] *EBF1* (early B-cell factor) is known to be a master regulator for B-cell development, as *Ebf1*-deficient mice produce only B-biased progenitor cells but not mature B cells. In addition, *EBF1*, by remodeling chromatin at the *PAX5* locus, is required for

PAX5 expression; in turn, *PAX5* controls the commitment of the common lymphoid progenitor to the B-cell pathway by repressing genes inappropriate for the B lineage and by activating genes required for B-cell maturation[25]. All known *PAX5* aberrations were shown to reduce the transcriptional activity of *PAX5* and its downstream targets. However, neither *EBF1* nor *PAX5* lesions demonstrate prognostic significance.[26]

IKZF1, CRLF2, JAK, IL7R, and the "*BCR-ABL1*-like" Signature

Recurrent deletions and inactivating mutations in the B-cell development gene *IKZF1* were also identified by Mullighan and associates.[24,26] *IKZF1* encodes the zinc finger lymphoid transcription factor IKAROS (see Figure 26-3, *B*). Isoforms of *IKZF1*, which lack N-terminal zinc fingers but retain the ability to dimerize, act as dominant negative inhibitors of IKAROS function and have been shown in murine models to be leukemogenic. Although thought to exist in only 7% of pediatric and 19% of adult ALL patients, the incidence is higher in high-risk ALL populations (29%)—especially in those carrying the t(9;22) Philadelphia chromosome, where they are present in over 80% of pediatric and 63% of adult patients.[27] In addition, *IKZF1* deletions are observed at the progression of CML to lymphoid blast crisis (but not in CML chronic phase) and are therefore thought to be central in the pathogenesis of *BCR-ABL1* lymphoid leukemia. It was also noted that *IKZF1* mutations were highly correlated with (but not pathognomonic for) a "*BCR-ABL1*-like" gene expression signature even in *IKZF1* mutant ALL lacking the t(9;22).[26,28,29]

Several other recently identified mutations also give rise to a "*BCR-ABL1*-like" signature: *CRLF2* alterations, *JAK* mutations (see Figure 26-3, *C*), and *IL7R* mutations.[29] *CRLF2* forms part of a heterodimeric complex with the interleukin-7 receptor alpha (IL7RA) to serve as the type I cytokine receptor for thymic stromal lymphopoietin (TSLP), a ligand that mediates B-cell precursor proliferation and survival through its activation of downstream JAK/STAT and PI3K/mTOR pathways. Several alterations of *CRLF2* have been noted in ALL, all of which lead to *CRLF2* overexpression, including a focal interstitial deletion of the pseudoautosomal region of the sex chromosomes that creates the *P2RY8-CRLF2* fusion, a translocation of *CRLF2* to the immunoglobulin heavy-chain transcriptional enhancer (*IGH@-CRLF2*) and the *CRLF2* F232C point mutation (see Figure 26-3, *D*).[30-32] *CRLF2* alterations have been noted in 5% to 8% of pediatric[29] and 10% to 15% of adult ALL, and with a higher incidence in high-risk ALL, in patients of Hispanic/Latino ethnicity, and in patients with Down syndrome (DS). *IL7R* gain-of-function mutations are seen in 6% to 10% of ALL and are noted in both B and T phenotypes.[33] *CRLF2* aberrations generally occur in cases lacking classic cytogenetic abnormalities, demonstrate a "*BCR-ABL1*-like" gene expression signature, and tend to co-occur with *IKZF1* and/or *JAK* mutations.[27,34] *CRLF2* has been shown to be a negative prognostic indicator in patients with NCI high-risk disease but not in those with standard-risk disease or in those with DS.

JAK2 mutations were initially identified as occurring in approximately 20% of DS ALL,[35] and *JAK* (mostly, but not exclusively *JAK2*) mutations were subsequently identified in 10% of a high-risk non-DS cohort.[36] Most are point mutations at R683, a site distinct from the V617F mutations frequently observed in polycythemia vera and other myeloproliferative neoplasms, but also within the *JAK2* pseudokinase domain and resulting in constitutive kinase activity. Not surprisingly, 70% of the *JAK*-mutated cases contained concomitant *IKZF1* deletions, and all cases clustered with the *BCR-ABL1* gene expression signature and had a poor outcome.[36] Several studies have demonstrated in vitro sensitivity to *JAK* inhibitors in *JAK*-mutated ALL. This has served as the rationale for an ongoing Phase I study of the JAK1/2 inhibitor ruxolitinib.

Secondary to the complex interactions among *IKZF1*, *CRLF2*, *JAK*, the "*BCR-ABL1*-like" gene expression sig *Nature*, and NCI risk status, it has been difficult to determine which factors retain independent prognostic significance. However, in a recent multivariate analysis, NCI high-risk status, positive end-induction minimal residual disease (MRD), high *CRLF2* expression, and *IKZF1* lesions had an independent adverse impact on relapse-free survival, and subgroup analyses demonstrated that high *CRLF2* expression was associated with poorer relapse-free survival in NCI high-risk but not standard-risk disease.[37]

CREBBP and Relapsed ALL

CREBBP (CREB binding protein or CBP) encodes for a large ubiquitously expressed protein of the same name that performs multiple roles in transcriptional coactivation, including the acetylation of histone and nonhistone targets. Germline mutations in *CREBBP* are observed in Rubenstein-Taybi syndrome, a developmental disorder characterized by dysmorphology, intellectual impairment, and increased susceptibility to solid tumors, and somatic mutations have recently been noted in more than one third of diffuse large-cell non-Hodgkin lymphoma and follicular lymphoma at diagnosis. Aberrations of *CREBBP* are exceedingly rare in de novo ALL; however, 19% of relapsed samples harbor loss-of-function mutations. Functionally, mutations impair histone acetylation and transcriptional regulation of *CREBBP* targets, including glucocorticoid-responsive genes.[38] Contemporaneous work identified a relapse-specific epigenetic profile characterized by global CpG island hypermethylation,

upregulation of genes involved in regulation of the cell cycle, and apoptosis and downregulation of genes involved in sensitivity to thiopurines, alkylators, and glucocorticoids. Along with in vitro studies demonstrating a chemosensitizing effect of histone deacetylase inhibitors and DNA methyltransferase inhibitors on relapsed patient samples, these data have formed the rationale for a recently opened Phase I trial of vorinostat and decitabine added to an intensive chemotherapy backbone in patients with relapsed ALL.

T ALL

T ALL makes up 12% of pediatric and 25% of adult ALL. It occurs most often in adolescents and young adults, frequently presenting with an extremely high WBC, CNS involvement, a mediastinal mass, marked lymphadenopathy, and hepatosplenomegaly. Historically, survival was dismal compared to B-lineage ALL, but with intensified therapies it has improved to approximately 80% in pediatrics[1] and 50% in adults. However, traditional risk factors such as age and WBC used for stratification in B-lineage ALL appear not to be as prognostically informative in T ALL, highlighting the importance of identifying molecularly based prognostic differences instead.

Although genetic translocations have been identified in T ALL (e.g., *MLL-ENL*, *CALM-AF10*, *ETV6-JAK2*, *ETV6-ABL1*, *EML1-ABL1*, and *NUP214-ABL1*), the leukemogenic event typically involves overexpression of an unaltered proto-oncogene, rather than generation of a novel fusion protein, due to a translocation placing it under control of a T-cell receptor (TCR) promoter or enhancer, most often TCRβ or TCRα/δ. The breakpoints occur at junctions where RAG recombinase acts during normal V(D)J recombination, suggesting they are the consequence of physiologic gene rearrangement processes gone awry.[39]

Numerous transcription factors have been identified as aberrantly expressed through juxtaposition to TCR loci in T ALL. They include MYC and several homologous bHLH proteins: TAL1, TAL2, LYL1, and BHLHB1. In addition, homeobox genes and components of the TCR signaling pathway such as SRC-family tyrosine kinases, *LCK*, and *NRAS* appear to be important targets of dysregulation in T ALL.

Aberrant *TAL1* expression via a variety of mechanisms occurs in more than 60% of T ALL. The oncogenic activity of *TAL1* seems to occur through sequestration and inactivation of *E2A* within a binding complex. LMO1 and LMO2 are transcription factors that play important roles in hematopoiesis and vascular development, serving as an interface for binding of multiple transcription factors in a large complex. TAL1 forms a heterodimeric DNA-binding complex with either LMO1 or LMO2, which cooperate in leukemogenesis at least in part through their inhibition of *E2A*.

A disturbing confirmation of the oncogenicity of LMO2 occurred during a gene therapy trial for X-linked severe combined immunodeficiency (SCID) caused by γ-chain deficiency.[40,41] Patients underwent retrovirus-mediated γ-chain gene transfer into autologous bone marrow progenitor cells. Approximately 3 years later, 2 of the 10 patients developed T ALL. The apparent mechanism was retroviral insertional mutagenesis. In both cases, the retroviral particle integrated close to the LMO2 locus and presumably exerted enhancer activity on the *LMO2* promoter, causing its overexpression in lymphoblasts.

NOTCH

The heterogeneous molecular genetics of T ALL was partially unified in 2004 with the discovery of a single gene upregulated in more than 50% of cases in both children and adults.[42] The gene, *NOTCH1*, is a regulatory transmembrane receptor that plays a crucial developmental role in cell fate determination and pattern formation, and in hematopoietic stem-cell maintenance and T-cell fate specification in the mature organism. *NOTCH1* was first identified in the rare T ALL translocation t(7;9)(q34;q34.3), which juxtaposes it to the TCRβ locus, leading to overexpression of a constitutively activated, truncated protein.

Poor prognostic features in T ALL include CD10 positivity, pro-T immunophenotype, and possibly *TAL1* expression. *HOX11* is generally associated with a favorable outcome, at least with modern intensive therapy. *HOX11L2* was reported in some studies as a poor prognostic feature, but intensive therapy appears to eliminate this effect. Cases with the *NUP214-ABL1* fusion appear aggressive and may benefit from imatinib. MRD positivity is a significant adverse prognostic marker in T ALL, occurring more often and correlating more closely with relapse compared to B-lineage ALL. Data suggest that *NOTCH1* mutations may be associated with a very favorable prognosis.[43]

Compound 506U, also known as nelarabine, is a nucleoside analog preferentially accumulated in T ALL that is being incorporated in current clinical trials. Since mutated *NOTCH1* activity depends on γ-secretase activity, γ-secretase inhibitors are also being investigated for therapeutic use.

Early T-Cell Precursor Phenotype (ETP)

A minority of T-ALL cases has recently been noted to carry a gene expression profile reminiscent of normal T-cell precursors, a subset of cells early in the process of thymic maturation that retain multilineage differentiation potential.[44] Consistent with a leukemogenic block early in development, ETP leukemia is defined by the lack of T-cell markers CD1a

and CD8, dim or absent CD5 expression on flow cytometric analysis, and the retention of one of a number of myeloid and/or hematopoietic stem-cell markers. The incidence is estimated at 15% in pediatric and 23% in adult T-ALL cases. Prognosis for ETP leukemia is very poor on conventional therapy, with a 10-year overall survival of 19% to 35%.[44] ETP exhibits a high burden of DNA copy number alterations (a mean of 14.1 per case vs. 6.3 per case in non-ETP T-cell ALL and 6.4 per case for all ALL), indicating a high degree of relative genomic instability[44]. Although no unifying genetic lesion has been identified, the ETP gene expression signature shows significant positive enrichment for genetic abnormalities expressed in leukemic stem cells and in AML cases with a poor outcome, such as cytokine receptor, *RAS*, and epigenetic modification.[45] These observations have raised the possibility that ETP should be treated with therapies known to be effective in AML and/or therapies known to inhibit cytokine receptor signaling.

ERG

The v-ets erythroblastosis virus E26 oncogene homolog (*ERG*) gene, at band 21q22, is a member of the ETS family of transcription factors, which are downstream effectors of mitogenic signaling transduction pathways and are involved in key steps in the regulation of cell proliferation, apoptosis, and differentiation. *ERG* is frequently expressed at high levels in leukemia. In fact, it has recently been shown that forced expression of ERG in adult bone marrow cells alters differentiation and induces expansion of T and erythroid cells; furthermore, the expanded T cells develop T ALL after acquisition of *NOTCH1* gene mutations in experimental models.[46] *ERG* overexpression has been shown to have adverse prognostic impact in both AML and T ALL.

Other ALL Aberrations

c-MYC, t(8;14)(q24;q32)

One of the first links between a chromosomal translocation, oncogene overexpression, and development of a human cancer was the discovery of *c-MYC* dysregulation in Burkitt's lymphoma in the early 1980s. The t(8;14)(q24;q32) translocation places *c-MYC* on chromosome 8 under control of the immunoglobulin heavy-chain gene on chromosome 14, resulting in constitutive *c-MYC* overexpression. *MYC* dysregulation is also an essential feature of mature B-cell leukemia, also known as Burkitt's leukemia. In essence, Burkitt's lymphoma and mature B-cell leukemia are best viewed as two manifestations of a common disease, differing only in the extent of dissemination. The similarity of their symptomatology, prognosis, and treatment illustrates the importance of a common underlying genetic mechanism in defining disease biology.

More than 90% of mature B-cell ALL exhibits the t(8;14) translocation. In the remainder, t(2;8)(p12;q24) or t(8;22)(q24;q11) places *MYC* under the control of the κ or λ light chains, respectively. Another variant, t(8;14)(q24;q11), involves the TCRα locus and has been reported in association with T ALL. Outcome for mature B-cell ALL has improved substantially since the shift from standard ALL therapy to much shorter (roughly 8-month duration), more dose-intensive regimens containing not only traditional ALL chemotherapy such as prednisone, vincristine, doxorubicin, cyclophosphamide, and high-dose methotrexate but additional drugs such as Ara-C, etoposide, and most recently rituximab.

7p Deletions and Monosomy 7

Losses involving chromosome 7 are more common in AML, but they do occur in approximately 5% of adult and pediatric ALL.[47] In adult ALL, deletion or loss of chromosome 7 is often associated with Ph positivity and does not have independent prognostic significance. In pediatric ALL, chromosome 7 losses tend to occur in patients with other concomitant adverse risk factors, but retain some independent negative prognostic impact.

9p21 Deletion

Deletions in the 9p21-22 region occur in 10% to 30% of ALL and more than 50% of T ALL, with the incidence rising as cytogenetics laboratories adopt more sensitive detection methods.[48] The principal targets appear to be the *INK4A* and *INK4B* loci, which contain two cyclin D kinase inhibitors, p16 and p15, that prevent abnormal cells from passing through the G_1 cell cycle checkpoint and are mutated, deleted, or epigenetically silenced in many cancers. 9p21 deletion often coincides with other adverse factors; its independent significance is controversial.

Cooperating Pathways: *p53, FLT3, Ras, PTPN11*

p53 is a classic tumor suppressor gene that initiates cell cycle arrest or apoptosis in response to abnormal proliferation, hypoxia, or DNA damage and is mutated in many human cancers. Mutation of p53 itself is rare in ALL, but mutations of elements of the pathway such as p14(ARF) and p21(CIP1) are common and may be associated with an unfavorable prognosis. FLT3 (for FMS-like tyrosine kinase) is a membrane-bound receptor tyrosine kinase involved in hematopoietic proliferation, differentiation, and apoptosis. Mutations resulting in ligand-independent activation are oncogenic and occur in 15% to 35% of AML and 1% to 3% of ALL.[49] *FLT3* mutations and FLT3 overexpression in ALL are particularly associated with *MLL*-r and hyperdiploidy. Ras family members play crucial signaling roles in proliferation, antiapoptosis, and other processes. Mutations in *NRAS* and *KRAS* have been linked to parental

exposures to certain drugs and hydrocarbons. Activating mutations of *PTPN11*, which encodes the tyrosine phosphatase SHP-2, enhance Ras signaling. Forty-five percent of *ETV6-RUNX1*-negative cases of pre-B ALL have mutations in *PTPN11*, *NRAS*, and/or *KRAS2*.[50] *RAS* mutations do not appear prognostically significant, whereas the significance of *PTPN11* mutations remains unclear. Recently, mutations in p53 and in the Ras and Pi3K signaling pathways were identified in hypodiploid ALL, with approximately half of the p53 mutations being germline (i.e., a manifestation of Li-Fraumeni syndrome).

Clinical Implications of Genetic Lesions in ALL

At diagnosis, the main adverse features currently being used for treatment decisions in pediatric ALL are *BCR-ABL1*, *MLL*-r, iAMP21, hypodiploidy (fewer than 44 chromosomes), and *MYC* rearrangements. Favorable features are *ETV6-RUNX1* and high hyperdiploidy or the presence of the particular trisomies 4 and 10. In adults, *BCR-ABL1* and *MLL*-r may affect treatment choices, whereas the other abnormalities are too rare or uncertain in impact to be routinely assessed or acted on.

Detection of molecular abnormalities in ALL has important implications for initial risk assignment as well as for disease monitoring. The classical definition of remission is less than 5% lymphoblasts in the bone marrow assessed by morphologic examination. However, newer molecular definitions of remission based on MRD detection are far more sensitive in identifying patients at risk of ultimate morphologic relapse. MRD can be measured by flow cytometry or PCR and sensitivity ranges from 10^{-3} to 10^{-6}. Numerous pediatric studies have demonstrated that MRD positivity has poor prognostic impact independent of other risk factors, at time points ranging from early induction to 24 months into treatment. Conversely, rapid achievement of MRD negativity before the end of induction identifies a favorable risk group that might be spared the adverse effects of high-intensity regimens. Adults tend to have higher MRD levels and more frequent and prolonged duration of MRD positivity—not surprisingly, given the comparative drug resistance of their disease. Nevertheless, MRD does appear to have independent predictive value in the adult population as well.

Conclusions

Unraveling the molecular genetics of ALL has paved the way toward many advances in our understanding of leukemogenesis, including improving our ability to stratify patients by risk group at diagnosis, tracking disease status during treatment, and identifying novel therapeutic targets. On a practical level, however, the recent explosion in the quantity of molecular genetic information regarding ALL poses an increasing challenge to those attempting to determine which abnormalities can best be exploited in current and future clinical trials.

Key References

1. Hunger SP, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *J Clin Oncol*. 2012;30(14):1663-1669.
2. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. *J Clin Oncol*. 2011;29(5):532-543.
3. Rabbitts TH. Chromosomal translocations in human cancer. *Nature*. 1994;372(6502):143-149.
4. Zhang J, et al. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood*. 2011;118(11):3080-3087.
5. Ferrando AA, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
8. Heerema NA, et al. Prognostic impact of trisomies of chromosomes 10, 17, and 5 among children with acute lymphoblastic leukemia and high hyperdiploidy (> 50 chromosomes). *J Clin Oncol*. 2000;18(9):1876-1887.
9. Bhojwani D, et al. ETV6-RUNX1-positive childhood acute lymphoblastic leukemia: improved outcome with contemporary therapy. *Leukemia*. 2012;26(2):265-270.
10. Raimondi SC, et al. Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognostic implications of the t(1;19). *J Clin Oncol*. 1990;8(8):1380-1388.
13. Schultz KR, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *J Clin Oncol*. 2009;27(31):5175-5181.
14. Druker BJ, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*. 2001;344(14):1038-1042.
17. Marschalek R. Mechanisms of leukemogenesis by MLL fusion proteins. *Br J Haematol*. 2011;152(2):141-154.
18. Ferrando AA, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
19. Bernt KM, Armstrong SA. Targeting epigenetic programs in MLL-rearranged leukemias. *Hematology Am Soc Hematol Educ Program*. 2011;2011:354-360.
23. Rand V, et al. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. *Blood*. 2011;117(25):6848-6855.
24. Mullighan CG, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758-764.
26. Mullighan CG, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360(5):470-480.
28. Den Boer ML, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol*. 2009;10(2):125-134.
29. Harvey RC, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. *Blood*. 2010;116(23):4874-4884.

35. Bercovich D, et al. Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome. *Lancet*. 2008;372(9648):1484-1492.
38. Mullighan CG, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature*. 2011;471(7337):235-239.
39. Rabbitts TH. Chromosomal translocation master genes, mouse models and experimental therapeutics. *Oncogene*. 2001;20(40):5763-5777.
42. Weng AP, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269-271.
44. Coustan-Smith E, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol*. 2009;10(2):147-156.
45. Zhang J, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. 2012;481(7380):157-163.
50. Tartaglia M, et al. Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. *Blood*. 2004;104(2):307-313.

References

1. Hunger SP, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *J Clin Oncol*. 2012;30(14):1663-1669.
2. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. *J Clin Oncol*. 2011;29(5):532-543.
3. Rabbitts TH. Chromosomal translocations in human cancer. *Nature*. 1994;372(6502):143-149.
4. Zhang J, et al. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Blood*. 2011;118(11):3080-3087.
5. Ferrando AA, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
6. Raimondi SC, et al. Near-triploidy and near-tetraploidy in childhood acute lymphoblastic leukemia: association with B-lineage blast cells carrying the ETV6-RUNX1 fusion, T-lineage immunophenotype, and favorable outcome. *Cancer Genet Cytogenet*. 2006;169(1):50-57.
7. Attarbaschi A, et al. Near-tetraploidy in childhood B-cell precursor acute lymphoblastic leukemia is a highly specific feature of ETV6/RUNX1-positive leukemic cases. *Genes Chromosomes Cancer*. 2006;45(6):608-611.
8. Heerema NA, et al. Prognostic impact of trisomies of chromosomes 10, 17, and 5 among children with acute lymphoblastic leukemia and high hyperdiploidy (> 50 chromosomes). *J Clin Oncol*. 2000;18(9):1876-1887.
9. Bhojwani D, et al. ETV6-RUNX1-positive childhood acute lymphoblastic leukemia: improved outcome with contemporary therapy. *Leukemia*. 2012;26(2):265-270.
10. Raimondi SC, et al. Cytogenetics of pre-B-cell acute lymphoblastic leukemia with emphasis on prognostic implications of the t(1;19). *J Clin Oncol*. 1990;8(8):1380-1388.
11. Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogenesis. *Oncogene*. 2001;20(40):5708-5717.
12. Raimondi SC, et al. New recurring chromosomal translocations in childhood acute lymphoblastic leukemia. *Blood*. 1991;77(9):2016-2022.
13. Schultz KR, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. *J Clin Oncol*. 2009;27(31):5175-5181.
14. Druker BJ, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med*. 2001;344(14):1038-1042.
15. Mathisen MS, et al. Role of tyrosine kinase inhibitors in the management of Philadelphia chromosome-positive acute lymphoblastic leukemia. *Curr Hematol Malig Rep*. 2011;6(3):187-194.
16. Biondi A, et al. Biological and therapeutic aspects of infant leukemia. *Blood*. 2000;96(1):24-33.
17. Marschalek R. Mechanisms of leukemogenesis by MLL fusion proteins. *Br J Haematol*. 2011;152(2):141-154.
18. Ferrando AA, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
19. Bernt KM, Armstrong SA. Targeting epigenetic programs in MLL-rearranged leukemias. *Hematology Am Soc Hematol Educ Program*. 2011;2011:354-360.
20. Spector LG, et al. Maternal diet and infant leukemia: the DNA topoisomerase II inhibitor hypothesis: a report from the children's oncology group. Cancer Epidemiol. *Biomarkers Prev*. 2005;14(3):651-655.
21. Taketani T, et al. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. *Blood*. 2004;103(3):1085-1088.
22. Attarbaschi A, et al. Minimal residual disease values discriminate between low and high relapse risk in children with B-cell precursor acute lymphoblastic leukemia and an intrachromosomal amplification of chromosome 21: the Austrian and German acute lymphoblastic leukemia Berlin-Frankfurt-Munster (ALL-BFM) trials. *J Clin Oncol*. 2008;26(18):3046-3050.
23. Rand V, et al. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. *Blood*. 2011;117(25):6848-6855.
24. Mullighan CG, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758-764.
25. Pongubala JM, et al. Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nat Immunol*. 2008;9(2):203-215.
26. Mullighan CG, et al. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med*. 2009;360(5):470-480.
27. Moorman AV, et al. IGH@ Translocations, CRLF2 Deregulation, and Microdeletions in Adolescents and Adults With Acute Lymphoblastic Leukemia. *J Clin Oncol*. 2012.
28. Den Boer ML, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *Lancet Oncol*. 2009;10(2):125-134.
29. Harvey RC, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. *Blood*. 2010;116(23):4874-4884.
30. Mullighan CG, et al. Rearrangement of CRLF2 in B-progenitor- and Down syndrome-associated acute lymphoblastic leukemia. *Nat Genet*. 2009;41(11):1243-1246.
31. Russell LJ, et al. Deregulated expression of cytokine receptor gene, CRLF2, is involved in lymphoid transformation in B-cell precursor acute lymphoblastic leukemia. *Blood*. 2009;114(13):2688-2698.
32. Yoda A, et al. Functional screening identifies CRLF2 in precursor B-cell acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 2010;107(1):252-257.
33. Shochat C, et al. Gain-of-function mutations in interleukin-7 receptor-alpha (IL7R) in childhood acute lymphoblastic leukemias. *J Exp Med*. 2011;208(5):901-908.
34. Harvey RC, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. *Blood*. 2010;115(26):5312-5321.
35. Bercovich D, et al. Mutations of JAK2 in acute lymphoblastic leukaemias associated with Down's syndrome. *Lancet*. 2008;372(9648):1484-1492.
36. Mullighan CG, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*. 2009;106(23):9414-9418.
37. Chen IM, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. *Blood*. 2012;119(15):3512-3522.
38. Mullighan CG, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. *Nature*. 2011;471(7337):235-239.
39. Rabbitts TH. Chromosomal translocation master genes, mouse models and experimental therapeutics. *Oncogene*. 2001;20(40):5763-5777.
40. Hacein-Bey-Abina S, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2003;348(3):255-256.
41. Hacein-Bey-Abina S, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302(5644):415-419.
42. Weng AP, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269-271.
43. Breit S, et al. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. *Blood*. 2006;108(4):1151-1157.
44. Coustan-Smith E, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol*. 2009;10(2):147-156.
45. Zhang J, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. 2012;481(7380):157-163.
46. Tsuzuki S, Taguchi O, Seto M. Promotion and maintenance of leukemia by ERG. *Blood*. 2011;117(14):3858-3868.
47. Heerema NA, et al. Deletion of 7p or monosomy 7 in pediatric acute lymphoblastic leukemia is an adverse prognostic factor: a report from the Children's Cancer Group. *Leukemia*. 2004;18(5):939-947.
48. Heerema NA, et al. Association of chromosome arm 9p abnormalities with adverse risk in childhood acute lymphoblastic leukemia: A report from the Children's Cancer Group. *Blood*. 1999;94(5):1537-1544.
49. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer*. 2003;3(9):650-665.
50. Tartaglia M, et al. Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. *Blood*. 2004;104(2):307-313.

David Malkin

27

Molecular Biology of Childhood Neoplasms

Cancer is the most common cause of disease-related death in children beyond the newborn period. Although childhood cancers, as a group, account for only a small proportion of all human cancer,[1] their unique biologic features, cell of origin, and response to therapy make them intriguing models with which to study and understand the process of human carcinogenesis.

Although most childhood cancers occur sporadically and their etiology remains unclear, hereditary or familial factors are evident in 25% to 40% of cases.[2] Obvious environmental influences on cancer initiation are not generally apparent. Cancer predisposition syndromes manifesting in childhood in which nonmalignant phenotypic features are not observed include hereditary retinoblastoma (RB), Li-Fraumeni syndrome, and familial polyposis; others such as von Hippel–Lindau disease and Gorlin syndrome are associated with the coincident presentation of both benign and malignant neoplasms. Nonrandom molecular and cytogenetic alterations are frequently observed in most childhood cancers. These "markers" provide not only unique diagnostic identifiers but also frequently prognostic value with respect to disease outcome and anticipated response to therapy. Many of these genetic markers also recapitulate normal developmental processes and thus offer a window into the biologic mechanisms of carcinogenesis and normal embryologic growth and development (Table 27-1). Importantly, the introduction of routine predictive genetic testing together with the development and implementation of clinical surveillance protocols has led to early tumor detection and improved survival for both children and adults with hereditary forms of cancer. In this chapter, we address the diversity of molecular mechanisms in several prototypical childhood cancers and cancer predisposition syndromes.

Retinoblastoma

Clinical Description and Pathology

Retinoblastoma (RB) is a rare childhood tumor thought to arise in the embryonic retinal epithelium. Although the incidence of RB is only approximately 1 in 20,000 births, or some 200 new cases per year in North America,[3] this tumor has been a target of intense research interest. RB is the prototype cancer caused by mutations of a tumor suppressor gene. Tumors are often bilateral and multifocal. In approximately 40% of RB cases, the disease is inherited as an autosomal dominant trait, with a penetrance approaching 100%.[4] The remaining 60% of cases are sporadic (nonheritable). Fifteen percent of unilateral RB is heritable but by chance develops in only one eye. Survivors of heritable retinoblastoma have a 100-fold increased risk of developing mesenchymal tumors such as osteogenic sarcoma, fibrosarcoma, and melanoma later in life. RB is characterized by the rapid growth of undifferentiated neuroblastic precursors derived from various layers of retinal ganglion cells. The cells are small and round with a high nuclear:cytoplasmic ratio, exhibiting numerous mitoses that reflect their rapid proliferative rate. RB cells appear undifferentiated, with evidence of ganglionic differentiation, including the presence of Flexner-Wintersteiner rosettes.

Tumors that are limited to the globe are staged according to the schema of Reese and Ellsworth, which is based on the number and size of the tumors.[5] These classification systems predict the likelihood of obtaining local tumor control and preservation of vision. Each eye is staged individually. RB can spread beyond the orbit by direct invasion of adjacent tissue and hematogenous spread. The treatment of patients with RB depends on the size of the tumor and the extent of tumor invasion at the time of diagnosis. Surgery is the mainstay of treatment for children with unilateral RB. Large intraocular tumors as well those with bilateral multiple tumors are treated with adjuvant chemotherapy.

Genetics and Cell Biology

The *RB* gene maps to chromosome 13q14.[6] Biallelic disruption of the *RB* gene leads to disease—an observation consistent with Knudson's "two-hit" theory of carcinogenesis.[4,7] *RB* consists of 27 exons and encodes pRB, a 105-kDa nuclear phosphoprotein plays a central role in the control of cell cycle

Table 27-1 Acquired Molecular and Cytogenetic Abnormalities in Childhood Neoplasms

Solid Tumor	Cytogenetic Abnormality	Genes*
Ewing sarcoma	t(11;22)(q24;q12),+8	*EWS(22) FLi1(11)*
Neuroblastoma	del1932–36, DMs, HSRs, +17q21-qter	*N-MYC*
Retinoblastoma	del13q14	*Rb*
Wilms tumor	del11p13, t(3;17)	*WT1*
Synovial sarcoma	t(X;11)(p11;q11)	*SSX(X) SYT(18)*
Osteogenic sarcoma	del13q14	?
Rhabdomyosarcoma	t(2;13)(q37;q14), t(1;13)(1p36;q14), 3p-, 11p-	*PAX3(2) FKHR(13); PAX7(1) FKHR(13)*
Peripheral neuroepithelioma	t(11;22)(q24;q12),+8	*EWS(22) FLi-14(11)*
Astrocytoma	i(17q)	?
Meningioma	delq22	*MN1, NF2, ?*
Atypical teratoid/rhabdoid tumor	delq22.11	*SNF5/INI1SMARCB1*
Germ cell tumor	i(12p)	?

*Chromosomal location in parentheses. ?, Gene unknown.

regulation, particularly in determining the transition from G_1 through S (DNA synthesis) phase in virtually all cell types.[8]

In the developing retina, inactivation of the *RB* gene is necessary and sufficient for tumor formation. It is now clear, however, that these tumors develop as a result of a more complex interplay of aberrant expression of other cell cycle control genes. In particular, a tumor surveillance pathway mediated by Arf, MDM2, MDMX, and p53 is activated after loss of pRB during development of the retina. In a small fraction of RB tumors, no *RB1* mutations are detected; in the majority of these, high-level amplification of the MYCN oncogene is observed, suggesting a novel mechanism of tumorigenesis in the presence of nonmutated *RB1* genes.[9] Not only do these observations provide a provocative biologic mechanism for tumor formation in retinoblastoma, but they also point to potential molecular targets for developing novel therapeutic approaches to this tumor. For example, the MDM2/MDMX antagonist, Nutlin-3a, efficiently targets the p53 pathway and is effective as an ocular formulation in treating RB in orthotopic xenografts.[10]

Wilms Tumor

Clinical Presentation and Pathology

Wilms tumor (WT), or nephroblastoma, is an embryonal malignancy that arises from remnants of immature kidney.[11] It affects approximately 1 in 7000 children, usually before the age of 6 years (median age at diagnosis, 3.5 years). Five percent to 10% of children present with synchronous or metachronous bilateral tumors. WT typically presents as an asymptomatic abdominal mass, although a small fraction of children have symptoms such as hematuria or hypertension. Approximately 20% of children present with metastatic disease.

The relationship between WT and aberrations of normal development is striking. In early development, the embryonal mesonephros emerges from a complex interaction between epithelial-derived ureteric bud tissue and mesenchymal-derived metanephric blastema through a series of differentiation events. Mature nephrons derived from the mesonephros are composed of nephroblasts, tubules, and stromal tissues that ultimately form the adult kidney. These different tissues together confer the distinctive "triphasic" histologic features of WT that arise in the intralobar area. Tumors that arise in the perilobar area tend to be biphasic or monomorphic, typically epithelial. This presentation suggests that these tumors arise from a cell that is more prevalent later in development, having a more limited potential to differentiate along multiple lineages.

A peculiar feature of WT is its association with nephrogenic rests, foci of primitive but nonmalignant cells whose persistence suggests a defect in kidney development. These precursor lesions are found within the normal kidney tissue of more than one third of children with WT. Nephrogenic rests may persist, regress spontaneously, or grow into large masses that simulate true WT and present a difficult diagnostic challenge.[11] Another intriguing feature of WT is its association with specific congenital abnormalities, including genitourinary anomalies, sporadic aniridia, mental retardation, and hemihypertrophy. A genetic predisposition to WT is observed in two distinct disease syndromes with urogenital system malformations—the WAGR (Wilms tumor, aniridia, genitourinary abnormalities, mental retardation) syndrome[12] and the Denys-Drash syndrome (DDS[13])—and in Beckwith-Wiedemann syndrome (BWS[14]). WT was the first of the solid tumors of childhood recognized as being curable even in the setting of metastatic disease. Sequential clinical treatment protocols evaluated by the National Wilms Tumor Study have led to effective multimodality approaches that cure up to 90% of children with WT. The cornerstone of therapy is surgery; chemotherapy agents with or without radiotherapy are used to treat minimal residual or metastatic disease.

Genetics

The WAGR syndrome is associated with constitutional deletions of chromosome 11q13.[12] Whereas it is now known that the WAGR deletion encompasses a number of

contiguous genes, including the aniridia gene *Pax6*, cytogenetic observation in patients with WAGR was also important in the cloning of the *WT1* gene at chromosome 11p13. *WT1* spans approximately 50 kb of DNA and contains 10 exons that encode the WT1 protein transcription factor. DDS, the second syndrome closely associated with this locus, is a rare association of WT, intersex disorders, and progressive renal failure.[13] Virtually all patients with DDS carry germline *WT1* point mutations.

WT1 is altered in only 10% of Wilms tumors. This observation implies the existence of alternative loci in the etiology of this childhood renal malignancy. One such locus also resides on the short arm of chromosome 11, telomeric of *WT1*, at 11p15. This gene, designated *WT2*, is associated with BWS. Patients with BWS are at increased risk of developing Wilms tumor, as well as other embryonic malignancies, including rhabdomyosarcoma (RMS), neuroblastoma, and hepatoblastoma.[14] The putative *BWS* gene maps to chromosome 11p15.[15] Whether the BWS gene and *WT2* are one and the same or two distinct yet closely linked genes remains to be determined. Using long-oligonucleotide array comparative genomic hybridization (array CGH), a novel gene termed *WTX* was identified on chromosome Xq11.1. *WTX* is inactivated in one third of WTs, and tumors with *WTX* mutations lack *WT1* mutations.[16] Bilateral WT or a family history of WT occurs in 1% to 5% of patients. Although linkage studies have indicated that the gene for familial WT must be distinct from *WT1* and *WT2*, and from the gene that predisposes to BWS, this gene has been neither cytogenetically localized nor isolated.

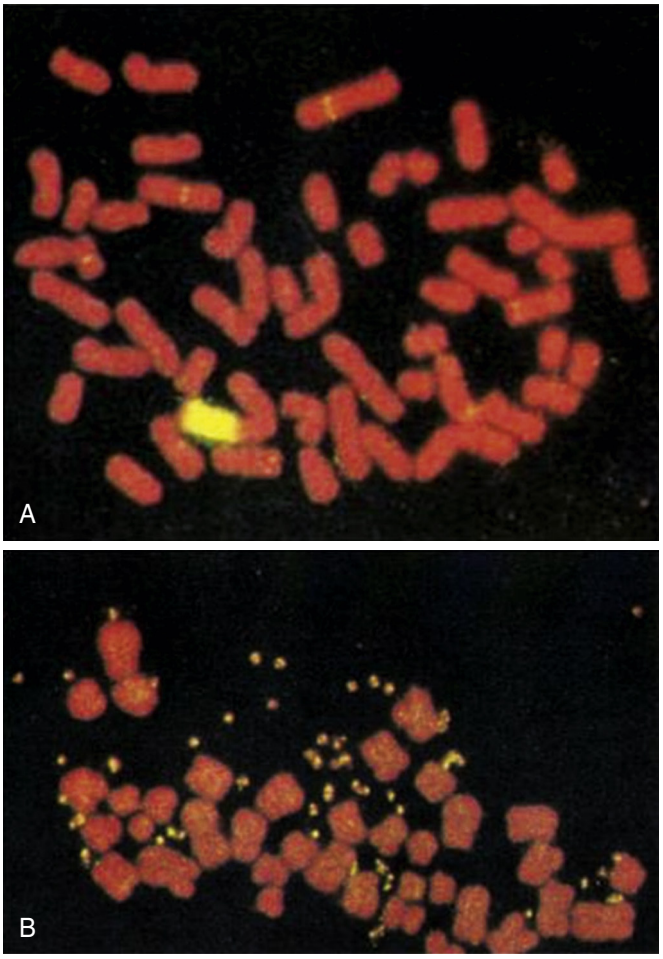


Figure 27-1 **A,** Homogeneously stained regions and, **B,**double-minute chromosomes in neuroblastoma.

Tumors of the Peripheral Nervous System: Neuroblastoma

Clinical Presentation and Pathology

Neuroblastoma (NB) is the most common tumor of the peripheral sympathetic nervous system in children. The embryonic neural crest gives rise to the peripheral nervous system including cranial and spinal sensory ganglia, autonomic ganglia, the adrenal medulla, and other para-endocrine cells distributed throughout the body.

NB most commonly arise in cells of the adrenal medulla and at other abdominal retroperitoneal sites of the known peripheral nervous system. Approximately 15% of cases occur in the paravertebral thoracic cavity in close association with the dorsal root ganglion. Most cases of NB (60% to 70%) present with metastatic disease, most commonly involving bone, bone marrow, and liver. NB is characterized histologically by the presence of small, round cells with hyperchromatic nuclei and stippled chromatin. At a cytogenetic level, homogeneously stained regions and double-minute chromosomes are typically observed (Figure 27-1). A hallmark of its light microscopic appearance is the presence of Homer-Wright rosettes characterized by tumor cell clusters around a central mesh of cell processes, termed *neuropile*.

The clinical stage and age of onset are highly significant independent prognostic variables. For example, a unique presentation of NB, stage IV-S (IV-special) is frequently associated with spontaneous remission.[17] This form of the disease typically presents in infants younger than 1 year of age with evidence of remote disease in the liver and bone marrow, though sparing bone. It is not known whether IV-S NB represents metastatic disease or a multifocal nonclonal disorder of neuroblast development. Stages I and II NB can generally be effectively managed with surgical resection alone, although those rare patients with low-stage disease and adverse biologic markers often require adjuvant chemotherapy. Multimodality therapy, including high-dose chemotherapy, hematopoietic stem cell harvest and rescue, radiation therapy, ^{123}I-MIBG therapy, and Ch14.18 immunotherapy are required to achieve remissions in stage IV (and to a lesser

extent stage III) NB, although remission is maintained in less than 40% of patients.

Genetics and Cell Biology

Nonrandom cytogenetic abnormalities are observed in more than 75% of neuroblastomas. The most common of these is deletion or rearrangement of the short arm of chromosome 1, although loss, gain, and rearrangements of chromosomes 10, 11, 14, 17, and 19 have also been reported. Two other unique cytogenetic rearrangements are highly characteristic of neuroblastoma: homogeneous staining regions and double-minute chromosomes (see Figure 27-1). These contain regions of amplification of the *N-myc* gene, an oncogene with considerable homology to the cellular proto-oncogene c-myc. *N-myc* amplification is associated with rapid tumor progression, and virtually all neuroblastoma tumor cell lines demonstrate amplified and highly expressed *N-myc*.[18] Decreased *N-myc* expression is observed in association with the in vitro differentiation of neuroblastoma cell lines.[19] This observation formed the basis for therapeutic trials demonstrating a survival advantage to patients treated with *cis*-retinoic acid.[20]

Neuroblastoma cells that express the high-affinity nerve growth factor receptor trkA can be terminally differentiated by nerve growth factor and demonstrate morphologic changes typical of ganglionic differentiation. Tumors showing ganglionic differentiation and *trkA* gene activation have a favorable prognosis. Expression of trkB receptor is associated with poor prognosis tumors and appears to mediate resistance to chemotherapy.

In addition to chromosomal loss on chromosome 1p36, unbalanced loss of heterozygosity at 11q23 is independently associated with decreased event-free survival. Alterations at 11q23 occur in almost one third of neuroblastomas, being most commonly associated with stage IV disease and age at diagnosis greater than 2.5 years. Telomerase expression and telomere length are yet other valuable markers of clinical significance.[21] In particular, short telomere length is predictive of favorable prognosis, regardless of disease stage, whereas long or unchanged telomeres are predictive of poor outcome. Both in vitro and in vivo studies suggest that telomerase inhibition may represent a unique mechanism for novel biological treatment of NB.[22] A small subset of neuroblastomas is inherited in an autosomal dominant fashion. Until recently, the only gene definitively associated with neuroblastoma risk was *PHOX2B*, also linked to central apnea.[23] Utilizing high-resolution microarray and next-generation sequencing approaches, de novo or inherited missense mutations in the tyrosine kinase domain of the ALK (anaplastic lymphoma kinase) gene on chromosome 2p23 have been observed in many hereditary neuroblastoma families, as well as in sporadic cases,[24] although no clear correlation with stage of disease has been observed. Current Phase I/II clinical trials with ALK inhibitors substantiate the value of such target identification for novel therapies. However, combination whole-exome, genome, and transcriptome sequencing of neuroblastoma identifies few recurrently mutated genes (*ALK*, *PTPN11*, *ATRX*, *MYCN*, and *NRAS*) or pathogenic germline variants (*ALK*, *CHEK2*, *PINK1*, and *BARD1*).[25]

Tumors of the Peripheral Nervous System: Ewing Sarcoma and Primitive Neuroectodermal Tumors

Clinical Description and Pathology

The Ewing sarcoma family of tumors (ESFT) make up the second most common bone malignancy after osteosarcoma in children and young adults with a peak incidence at age 15. Rarely, these tumors can arise in the soft tissues. ESFT includes Ewing sarcoma, peripheral primitive neuroectodermal (PPNET), and Askin tumors, among others. These tumors share indistinguishable genetic alterations, immunohistochemical profiles, and lineage-specific marker expression patterns. As in the case of neuroblastoma, therapy for patients with localized PPNET or ES is highly effective, whereas the prognosis for patients with metastatic disease is extremely poor even in the setting of multimodal therapy. Surgery and radiation are used for primary local control with multi agent chemotherapy used to treat systemic disease.

Genetics and Cell Biology

PPNET and ES typically carry a t(11;22)(q24;q12) chromosomal rearrangement, although variant translocations have been observed.[26] The translocation breakpoint has been molecularly cloned and characterized as an in-frame fusion between the 5′ half of the ES gene, *EWS*, on chromosome 22 and the 3′ half of the human homologue of an ETS transcription family member, *FLI1*, on chromosome 11. The resultant chimeric protein replaces the DNA-binding domain of EWS with the ETS-like binding domain of Fli-1, retaining the DNA-binding activity of Fli-1. Important transcriptional targets of the EWS-Fli1 transcription factor may include the IGF-I receptor,[27] which is thought to play a role in the pathogenesis of ES. Several studies have indicated the importance of the autocrine stimulation of the insulin-like growth factor I receptor (IGF-IR) for cell transformation and proliferation induced by EWS-Fli1.

Small-molecule inhibitors that block the EWS-Fli1 interaction with RNA helicase A showed promise in inhibiting Ewing sarcoma cell growth[28]; however, weak association of IGF-1R activity in ES cell lines and primary tumors indicate that it is not an ideal druggable target in this tumor.[29] Expression profiling analysis has also revealed that *TP53* is transcriptionally upregulated by the EWS-ETS fusion gene. *NKX2.2* has been found to be another target gene of EWS-Fli1 that is required for malignant transformation. Several variant translocations have also been identified, invariably fusing the *EWS* gene to an ETS family member. Interestingly, it has been suggested that the specific fusion protein expressed in ESFT has prognostic significance.[30] In particular, a rearrangement that joins exon 7 of *EWS* to exon 6 of *Fli1* may confer a more favorable outcome. As well, use of RNA sequencing (RNAseq) in EWS-FLI fusion negative ESFTs has identified at least one novel alteration that fuses *BCOR* (encoding the BCL6 co-repressor) on chromosome X 11p.14 with *CCNB3* (encoding the testis-specific cyclin B3) on chromosome X 11p.22, essentially identifying a novel sarcoma phenotype.[31]

Rhabdomyosarcoma

Clinical Description and Pathology

Sarcomas arise in supportive tissues that have their origin in embryonic mesenchyme. These tissues include fibrous tissue, muscle, cartilage, and bone. Each of the different sarcomas exhibits evidence of differentiation along one or more of these cellular lineages.

Rhabdomyosarcoma is the most common soft tissue sarcoma of childhood, with approximately 200 new cases annually in the United States, accounting for nearly 10% of all childhood solid tumors. The incidence of RMS is higher in males than in females (1.4:1), and most cases are diagnosed in children under the age of 6.[32] Rhabdomyosarcoma is believed to arise from primitive embryonic mesenchymal cells committed to the skeletal muscle lineage; however, RMS tumors have been found in tissues not usually containing striated muscle, such as the urinary bladder. Multiple histologic subtypes exist, including predominantly embryonal (ERMS; 63% of all tumors) and alveolar (ARMS; 19%) morphologies. ARMS tend to occur in the extremities and exhibit a more aggressive clinical behavior than ERMS, which tend to present in an axial distribution and with a somewhat more favorable prognosis. The management of RMS typically includes local control with both surgery and radiation treatment, with neo-adjuvant chemotherapy being used for management of known and micrometastatic disease. It is notoriously difficult to achieve a sustained remission, or cure, for children with metastatic RMS, particularly those with the alveolar variant. Although the overall survival for childhood RMS is approximately 75%, those with metastatic disease have a less than 20% chance of cure.

Genetics and Cell Biology

Characteristic genetic lesions have been found in both major subtypes of RMS. More than 75% of tumors of the alveolar subtype demonstrate one of two chromosomal translocations, t(2;13)(q35;q14) or t(1;13)(p36;q14),[33,34] which fuse the 5′ DNA-binding region of *PAX-3* on chromosome 2 or *PAX-7* on chromosome 1, respectively, which are implicated in neuromuscular development, to the 3′ transactivation domain region of the *FKHR (FOXO1A)* gene—a member of the forkhead family of transcription factors commonly associated with regulation of apoptosis (Figure 27-2). Tumors with the t(2;13) translocation have a much poorer prognosis than those with the rarer t(1;3) rearrangement. Interestingly, fusion-negative ARMS tumors are clinically and molecularly indistinguishable from embryonal RMS, and demonstrate outcomes more closely resembling ERMS than fusion-positive ARMS, thus making the presence of the PAX-FKHR fusion a diagnostic criterion for ARMS.[35] Additional epigenetic or genetic events seem required for RMS tumorigenesis. PAX-3-FKHR fusion is associated with increased expression of c-met. Met is the receptor tyrosine kinase for hepatocyte growth factor/scatter factor and is overexpressed in embryonal and alveolar RMS. Other frequently reported genetic alterations that may be common to embryonal and alveolar RMS include activated forms of *N-* and *K-RAS*, inactivating *TP53* mutations, and amplification and overexpression of *MDM2*, *CDK-4*, and *N-MYC*.

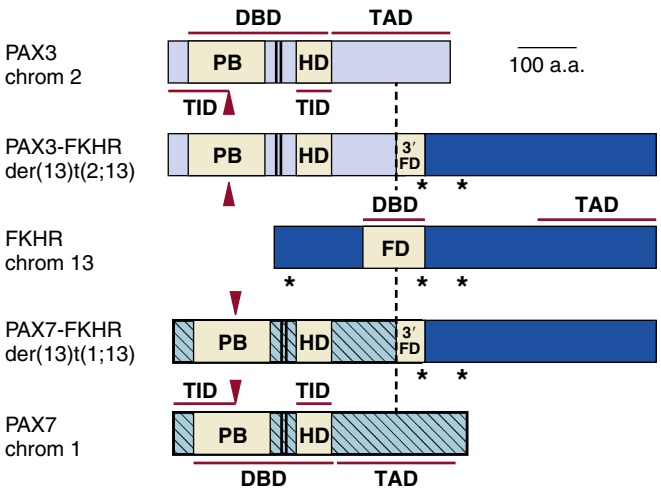


Figure 27-2 Translocation breakpoints in rhabdomyosarcoma.

At the molecular level, embryonal tumors are characterized by loss of heterozygosity (LOH) at the 11p15 locus, which is of particular interest because this region harbors the *IGF2* gene.[36] The LOH at 11p15 occurs by loss of maternal and duplication of paternal chromosomal material.[37] Although LOH is normally associated with loss of tumor suppressor gene activity, in this instance LOH with paternal duplication may result in activation of *IGF2*. This occurs because *IGF2* is now known to be normally imprinted—that is, this gene is normally transcriptionally silent at the maternal allele, with only the paternal allele being transcriptionally active. Thus, LOH with paternal duplication potentially leads to a twofold gene-dosage effect of the *IGF2* locus.

In addition to the somatic molecular changes associated with RMS, the tumor is also observed in hereditary cancer syndromes, including Li-Fraumeni syndrome (see following section), in which carriers harbor constitutional mutations of the *TP53* tumor suppressor gene. The possible importance of the patched gene, *PTCH*, in the development of RMS is suggested by the finding that mice lacking this gene develop RMS. *PTCH* is mutated in the germline of patients with Gorlin syndrome, a disorder that includes predisposition to tumor (medulloblastoma) development. Strikingly, *PTCH* is shown to regulate another gene, *GLI*, which is found to be amplified in RMS and Gorlin syndrome–associated tumors. Activation of the *HRAS* oncogene by heterozygous germline mutations predisposes to RMS in Costello syndrome,[38] further highlighting the multiple molecular pathways associated with rhabdomyosarcomagenesis.

Childhood Sarcomas: Osteosarcoma

Clinical Description and Pathology

Osteosarcoma (OS) occurs most frequently in adolescence during a period of rapid bone growth. It is the most common tumor in this age group other than those of hematopoietic tissues. OS most commonly occurs at metaphyseal growth plates of long bones, develops earlier in girls than in boys, and is more frequent in taller children. These observations suggest an important role for cellular proliferation in the oncogenic conversion of immature bone precursors from which these tumors are thought to arise. The histologic diagnosis of OS is made when tumor osteoid and disorganized bone can be identified within malignant stromal tissues. A wide range of histologic patterns is seen, although the natural history of these variants is not yet clinically distinguishable. Tumors are classified as osteoblastic, chondroblastic, or fibroblastic OS depending on whether the predominant differentiation is a long bone, cartilage, or stromal tissue pathway, respectively. Surgery is the primary therapeutic modality in the management of osteosarcoma. Neo-adjuvant chemotherapy is used to control micrometastases, which are present in 75% of patients. The response to chemotherapy, as measured by histologic grading of the degree of tumor necrosis, is a key prognostic factor. Biologic response modifiers, monoclonal antibodies, and targeted small-molecule kinase inhibitors have had no impact on the treatment of osteosarcoma.

Genetics and Cell Biology

OS is characterized by the presence of complex unbalanced karyotypes.[39] Combined inactivation of the RB1 and T*P53* tumor suppressor pathways are observed in most OS, indicating important roles for both these genes in OS pathogenesis. Further evidence for the role of p53 in OS pathogenesis includes the predisposition of patients with germline *TP53* mutations (Li-Fraumeni syndrome [LFS]) to develop OS. There is low prognostic significance of *TP53* mutations in sporadic OS, with no impact on distant recurrence. Furthermore, p53 status is concordant in paired samples of primary and distant metastases, suggesting that p53 pathway alterations may occur early in OS pathogenesis. Modifying effects of other expressed genes are being explored in OS. In particular, amplification of chromosome 12q13 region (containing *MDM2* and *CDK4*) or deletion of *INK4A* can disrupt both the p53 and RB pathways. Many recurrent, nonrandom chromosomal abnormalities are observed in OS. Common numerical abnormalities in OS include gain of chromosome 1; loss of chromosomes 9, 10, 13 (at the *RB1* locus), and/or 17 (at the *TPS3* locus); and partial or complete loss of the long arm of chromosome 6. Frequent structural abnormalities include rearrangements of chromosomes 11, 19, and 20. Genome-wide efforts to identify potential tumor suppressor genes associated with LOH in OS have identified at least one novel locus encompassing the *LSAMP* gene on chromosome 3p13.4.[40] Examination of 38 chromosomal arms from OS tumor samples for LOH has found that the mean frequency of LOH is 30.79% for any chromosome arm, an unusually high mean frequency for a childhood tumor. Moreover, several chromosome arms (3q, 13q, 17p, and 18q) underwent LOH with a frequency more than two standard deviations higher than the average ($p < 0.002$).[26] Further mitotic mapping has identified minimal regions thought to contain candidate tumor suppressor loci on chromosomal arms 3q26.2 and 18q21.33,[41] though specific gene identification has been elusive. Finally, an intriguing mechanism of chromosomal instability defined as "chromothripsis," in which chromosomal fragments undergo a catastrophic one-time rearrangement, has been observed in about 25% of osteosarcomas—far greater than the 2% to 3% observed in other human cancers.[42]

Abnormalities of bone growth and remodeling are thought to play an important role in the pathogenesis of OS. Normal bone repair involves proliferation of mesenchymally derived precursor cells mediated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), IGFs, interferon-α, and other mitogens. The PDGF receptor encodes a tyrosine kinase; when PDGF binds to its receptor, it induces expression of fos, myc, and a cascade of cellular genes important for initiating proliferation. EGF may also play a role in OS pathogenesis. Some OS cell lines express EGF receptor and proliferate in response to exposure to EGF. The mitogenic response to both EGF and PDGF may be blocked by TGF-β, which at low doses may stimulate the proliferation of cells.

Interest in other signaling pathways has implicated the Fas cell death pathway in determining chemosensitivity and metastatic behavior in OS.[43] Tumor cells expressing surface Fas will apoptose when Fas ligand (FasL) is present unless a mechanism of resistance is present. This has been suggested by studies in which metastasis-prone OS cell lines that have been transfected with Fas demonstrate reduced metastatic potential. In addition, overexpression of CyclinE1, which promotes oncogenic transformation of osteoblasts and confers resistance to cisplatin (a drug that forms the backbone of osteosarcoma therapy), offers new avenues for therapy.[44]

Cancer Predisposition Syndromes

Several hereditary cancer syndromes are associated with the occurrence of childhood as well as adult-onset neoplasms. Although it is beyond the scope of this chapter to describe them all, it is worthwhile to discuss a few to highlight the important molecular basis on which these disorders develop (Table 27-2).

Li-Fraumeni Syndrome

Li-Fraumeni familial cancer syndrome is the prototypical familial cancer predisposition syndrome. The definition of classical LFS requires a proband with a sarcoma diagnosed before 45 years of age, a first-degree relative diagnosed as having any cancer when younger than 45 years, and a first- or second degree relative with a diagnosis of cancer when younger than 45 years or a sarcoma at any age.[45] The classic spectrum of tumors that includes soft tissue sarcomas, osteosarcomas, breast cancer, brain tumors, leukemia, and adrenocortical carcinoma (ACC) has been overwhelmingly substantiated by numerous subsequent studies, although other cancers, usually of particularly early age of onset, are also observed. Similar patterns of cancer that do not meet the classic definition have been termed Li-Fraumeni–like syndrome (LFS-L). The sensitivity and specificity of the Chompret criteria are 82% and 58%, respectively, making it perhaps the most rigorous and relevant definition to justify *TP53* mutation analysis.[46]

Germline alterations of the *TP53* tumor suppressor gene are associated with LFS.[47,48] These are primarily missense mutations that yield a stabilized mutant protein. The spectrum of germline *TP53* mutations is similar to that of somatic mutations found in a wide variety of tumors. Carriers are heterozygous for the mutation, and in tumors derived from these individuals, the second (wild-type) allele is frequently deleted or mutated, leading to functional inactivation. Several comprehensive databases document all reported germline (and somatic) *TP53* mutations and are of particular value in evaluating novel mutations as well as phenotype-genotype correlations. Approximately 75% of "classic" LFS families have detectable *TP53* alterations. It is not clear whether the remainder are associated with the presence of modifier genes, promoter defects yielding abnormalities of p53 expression, or simply the result of weak phenotype-genotype correlations (i.e., the broad clinical definition encompasses families that are not actual members of LFS). The variability in type of cancer and age of onset within and between LFS families suggests that expression of modifier genes might influence the underlying mutant *TP53* genotype. Several of these have been described, including those that accelerate age of tumor onset in *TP53* mutation carriers such as a single-nucleotide polymorphism (SNP) in the promoter of the *MDM2* gene (SNP 309) that is involved in the p53 degradation pathway[49]; accelerated telomere attrition—perhaps inducing chromosomal chromothripsis in somatic cells[50]; and increased constitutional copy number variation (CNVs).[51] Others, such as a 16-bp duplication in *TP53* intron 3 (PIN3), delay tumor onset by up to 19 years.[52] Until recently, options for intervention in LFS were thought to be limited, but two studies have clearly demonstrated the value of total body imaging (with or without biochemical marker studies) in *TP53* mutation carriers (Table 27-3).[53,54] This has proven to be effective in early tumor detection, which leads to improved survival—offering hope for these patients that the combination of molecular testing with early clinical surveillance can interfere with the natural history of the disease.

Hereditary Paraganglioma Syndromes

Paragangliomas are benign non–catecholamine-secreting tumors that often occur in the head and neck region, along the parasympathetic chain. Catecholamine-secreting tumors can develop along the sympathetic chain, in the adrenal

Table 27-2 Hereditary Syndromes Associated with Childhood Neoplasms

Syndrome	OMIM Entry	Major Tumor Types	Mode of Inheritance	Genes
Hereditary Gastrointestinal Malignancies				
Adenomatous polyposis of the colon	175100	Colon, thyroid, stomach, intestine, hepatoblastoma	Dominant	*APC*
Juvenile polyposis	174900	Gastrointestinal	Dominant	*SMAD4/DPC4*
Peutz-Jeghers syndrome	175200	Intestinal, ovarian, pancreatic	Dominant	*STK11*
Genodermatoses with Cancer Predisposition				
Nevoid basal cell carcinoma syndrome	109400	Skin, medulloblastoma	Dominant	*PTCH*
Neurofibromatosis type 1	162200	Neurofibroma, optic pathway glioma, peripheral nerve sheath tumor	Dominant	*NF1*
Neurofibromatosis type 2	101000	Vestibular schwannoma	Dominant	*NF2*
Tuberous sclerosis	191100	Hamartoma, renal angiomyolipoma, renal cell carcinoma	Dominant	*TSC1/TSC2*
Xeroderma pigmentosum	278730, 278700, 278720, 278760, 278740, 278780, 278750, 133510	Skin, melanoma, leukemia	Recessive	*XPA, B, C, D, E, F, G, POLH*
Rothmund-Thomson syndrome	268400	Skin, bone	Recessive	*RECQL4*
Leukemia/Lymphoma Predisposition Syndromes				
Bloom syndrome	210900	Leukemia, lymphoma, skin	Recessive	*BLM*
Fanconi anemia	227650	Leukemia, squamous cell carcinoma, gynecological system	Recessive	*FANCA,B,C,D_2,E,F,G*
Schwachman-Diamond syndrome	260400	Leukemia/myelodysplasia	Recessive	*SBDS*
Nijemegen breakage syndrome	251260	Lymphoma, medulloblastoma, glioma	Recessive	*NBS_1*
Ataxia telangiectasia	208900	Leukemia, lymphoma	Recessive	*ATM*
Genitourinary Cancer Predisposition Syndromes				
Simpson-Golabi-Behmel syndrome	312870	Embryonal tumors, Wilms tumor	X-linked	*GPC3*
von Hippel–Lindau syndrome	193300	Retinal and central nervous hemangioblastoma, pheochromocytoma, renal cell carcinoma	Dominant	*VHL*
Beckwith-Wiedemann syndrome	130650	Wilms tumor, hepatoblastoma, adrenal carcinoma, rhabdomyosarcoma	Dominant	*CDKN1C/NSD1*
Wilms tumor syndrome	194070	Wilms tumor	Dominant	*WT1*
WAGR syndrome	194072	Wilms tumor, gonadoblastoma	Dominant	*WT1*
Costello syndrome	218040	Neuroblastoma, rhabdomyosarcoma, bladder carcinoma	Dominant	*H-Ras*
Central Nervous System Predisposition Syndromes				
Retinoblastoma	180200	Retinoblastoma, osteosarcoma	Dominant	*RB1*
Rhabdoid predisposition syndrome	601607	Rhabdoid tumor, medulloblastoma, choroid plexus tumor		*$SNF5/INI1$*
Medulloblastoma predisposition	607035	Medulloblastoma	Dominant	*SUFU*
Sarcoma/Bone Cancer Predisposition Syndromes				
Li-Fraumeni syndrome	151623	Soft tissue sarcoma, osteosarcoma, breast, adrenocortical carcinoma, leukemia, brain tumor	Dominant	*TP53*
Multiple exostosis	133700, 133701	Chondrosarcoma	Dominant	*EXT1/EXT2*
Werner syndrome	277700	Osteosarcoma, meningioma	Recessive	*WRN*

Table 27-2 Hereditary Syndromes Associated with Childhood Neoplasms—cont'd

Syndrome	OMIM Entry	Major Tumor Types	Mode of Inheritance	Genes
Endocrine Cancer Predisposition Syndromes				
MEN1	131000	Pancreatic islet cell tumor, pituitary adenoma, parathyroid adenoma	Dominant	*MEN1*
MEN2	171400	Medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia	Dominant	*RET*

OMIM, Online Mendelian Inheritance in Man.

Table 27-3 Clinical Criteria for Classic Li-Fraumeni Syndrome, LFS-Like Criteria, and Chompret Criteria

Classic LFS Criteria
Proband diagnosed with sarcoma before age 45
A first-degree relative with cancer diagnosed before age 45
A first- or second-degree relative on the same parental lineage with cancer diagnosed before age 45 or a sarcoma at any age
LFS-like Criteria (Birch)
Proband with any childhood cancer or sarcoma, brain tumor, or adrenocortical cancer diagnosed before age 45
First- or second-degree relative with typical LFS cancer (sarcoma, breast cancer, brain tumor, leukemia, or adrenocortical cancer) diagnosed at any age, AND
A first- or second-degree relative on the same side of the family with any cancer diagnosed under age 60
LFS-like Criteria (Eeles)
Two first- or second-degree relatives with LFS-related malignancies at any age
Chompret Criteria for LFS
Proband diagnosed with a narrow-spectrum cancer (sarcoma, brain tumor, breast cancer, or adrenocortical carcinoma) before age 46 and at least one first- or second-degree relative with any cancer, except breast cancer if the proband has breast cancer
Proband with multiple primary tumors, two of which belong to the narrow spectrum and the first of which occurred before age 46, regardless of family history
Proband with adrenocortical cancer or choroid plexus carcinoma, regardless of age at diagnosis or family history

medulla (pheochromocytoma) alongside the aortopulmonary vasculature, in the organ of Zuckerkandl, or even in the bladder and vas deferens. Paragangliomas have an estimated population incidence of 1 in 30,000. However, in the presence of an underlying germline succinyl dehydrogenase (*SDHx*) mutation, the tumor rate is extraordinarily high, with disease penetrance approaching 80%.[55,56] Germline *SHDx* mutations are identified in nearly 30% of patients with nonmetastatic paragangliomas and pheochromocytomas and in 44% of adults and 81% of pediatric patients with metastatic disease.[57] Other tumors, including renal cell carcinoma, oncocytoma, papillary thyroid cancer, pituitary tumors, gastrointestinal stromal tumor (GIST), and even neuroblastoma, are observed in *SDHx* mutation carriers. Although patients with localized asymptomatic disease are frequently observed, those with metastatic disease are particularly difficult to treat, often requiring multiple surgical procedures. Systemic chemotherapy is generally not effective, although the introduction of multitargeted kinase inhibitors provides a new avenue of molecularly targeted therapy.[58]

Genetics

Succinate dehydrogenase (SDH) is part of respiratory complex II in the mitochondrion, and this enzyme complex is responsible for converting succinate to fumarate as part of the Krebs cycle. SDH is composed of four distinct proteins called SDHA, SDHB, SDHC, and SDHD.[59] A fifth gene called *SDHAF2*, or SDH Assembly Factor 2, is responsible for assembling all of the individual SDH proteins into a fully functioning enzyme complex.[60] Germline mutations in each of these *SDHx* genes may lead to development of paragangliomas or pheochromocytomas. Lack of a functioning SDH complex leads to increased succinate, with subsequent increases in HIF signaling and possible histone deregulation. Germline mutations in other genes such as *NF1*, *VHL*, *RET*, *TMEM127*, and *MAX* also have been associated with the development of paragangliomas and pheochromocytomas (Figure 27-3). Based on gene expression and pathway analysis, these tumors can be divided into two different clusters that correspond to their underlying gene mutations: Cluster 1 (Cluster 1A: *SDHx*, Cluster 1B: *VHL*) associated with pseudohypoxia and aberrant VEGF signaling, and Cluster 2 (*RET/NF1/TMEM127/MAX*) associated with aberrant kinase signaling pathways.

The phenotype associated with each *SDHx* gene mutation leads to a different disease phenotype and clinical presentation, as outlined in Table 27-4.[59] To facilitate genetic diagnosis, risk assessment and treatment options, it is now possible to test for all the *SDHx* genes simultaneously.

Regular surveillance can detect early tumors in patients with underlying germline *SDHx* mutations. As with

surveillance in *TP53* mutation carriers, this is important so that smaller, asymptomatic SDH-deficient tumors can be removed before they transform to malignant and metastatic disease. Although no formalized screening guidelines exist, many clinicians will perform annual physical examinations, blood pressure checks (for hypertension due to increased catecholamines), and blood work for serum metabolites. Previously, urine catecholamines were examined from 24-hour urine specimens, but many clinicians have eliminated urine screening in favor of serum testing. Fractionated plasma metanephrines are the most sensitive and specific serum test for detecting secreting paragangliomas and pheochromocytomas.[61] Increased methoxytyramine, a metabolite of dopamine, seems to be helpful for predicting the likelihood of metastatic disease and for distinguishing SDH-related tumors from VHL-related tumors. However, testing of methoxytyramine remains difficult to obtain on a clinical basis.

Regular imaging has been demonstrated by several groups to be very effective at identifying SDH-related tumors, especially in the setting of negative biochemical results.[62] Screening approaches using rapid-sequence whole-body magnetic resonance imaging (MRI) in conjunction with urinary and or fractionated plasma metanephrine levels are being used widely. Abnormal MRI results (or biochemical results) are followed with positron emission tomography (PET) imaging to refine the anatomical location of the tumor.

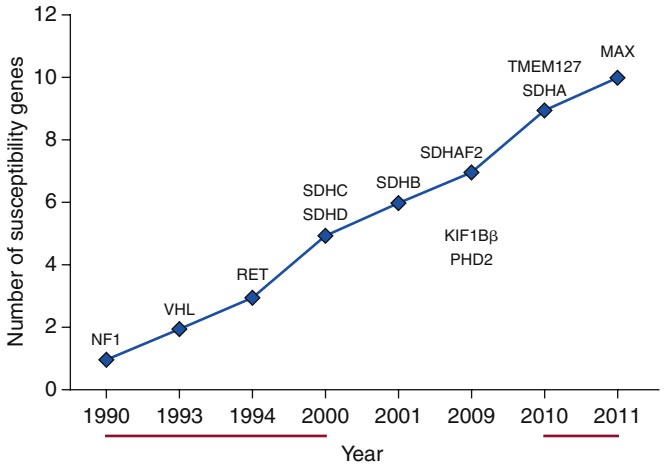


Figure 27-3 Accelerated discovery of genes associated with predisposition to hereditary pheochromocytoma/paraganglioma syndromes.

Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome occurs with a frequency of 1 in 13,700 births. BWS is associated with a wide spectrum of phenotypic stigmata, including hemihypertrophy/hemihyperplasia, exomphalos, macroglossia, gigantism, and ear pits (posterior aspect of the pinna). Laboratory findings may include profound neonatal hypoglycemia, polycythemia, hypocalcemia, hypertriglyceridemia, hypercholesterolemia, and high serum α-fetoprotein (AFP) level. With increasing age, phenotypic and laboratory features of BWS become less pronounced. Although neurocognitive defects are not universal in BWS, early diagnosis of the condition is crucial to avoid deleterious neurologic effects of neonatal hypoglycemia and to initiate an appropriate screening protocol for tumor development. The increased risk for tumor formation

Table 27-4 SDHX Genotype: Phenotype Correlations

	pgl1	pgl2	pgl3	pgl4	pglx
SDH gene	*SDHD*	*SDH5 (SDHAF2)*	*SDHC*	*SDHB*	*SDHA*
Chromosomal location	11q23	11q11.3	1q21	1p3536.1	5p15
First described	Baysal et al. (2000)[60a]	Hao et al. (2009)[60]	Nieumann and Muller[60b] (2000)	Astuti et al. (2001)[60c]	Burnichon et al. (2010)[60d]
Most common mutation	Frameshift	Point	Nonsense	Missense	Missense
Head and neck PGLs	++	++	++	+	+
PCC (any abdominal)	+/–	–	+/–	++	++
Catecholamine secreting	+/–	–	+/–	++	?
Malignant	–	–	Unknown	++	+
Associated with GIST	+	–	+	+	–
Associated with thyroid cancer	+	–	–	+	–
Associated with renal tumors (renal cell carcinoma and oncocytoma)	–	–	–	+	–
Associated with neuroblastoma	–	–	–	+	–

in BWS patients is estimated at 7.5% and is further increased to 10% if hemihyperplasia is present. Tumors occurring with the highest frequency include Wilms tumor, hepatoblastoma, neuroblastoma, rhabdomyosarcoma, and ACC.

The genetic basis of BWS is complex, and it does not appear that a single gene is responsible for the BWS phenotype. Various 11p15 chromosomal or molecular alterations have been associated with the BWS phenotype (Table 27-5) and its tumors.[63] Abnormalities in this region affect an imprinted domain, indicating that it is more likely that normal gene regulation in this part of chromosome 11p15 occurs in a regional manner and may depend on various interdependent factors or genes. These include the paternally expressed genes *IGF2* and *KCNQ10T1* and the maternally expressed genes *H19*, *CDKN1C*, and *KCNQ1*. Paternal uniparental disomy, in which two alleles are inherited from one parent (the father), has been reported in approximately 15% of sporadic BWS patients.[64] The insulin/IGF2 region is always represented in the uniparental disomy, although the extent of chromosomal involvement is highly variable. Alterations in allele-specific DNA methylation of *IGF2* and *H19* reflect this paternal imprinting phenomenon.[64] As with other cancer susceptibility syndromes, effective clinical surveillance protocols regularly identify tumors with demonstrable improved outcomes. Regular (every 3 months) AFP levels and abdominal/pelvic ultrasound are recommended until the affected child is about 9 years old and generally beyond the risk age for the associated tumors.

Gorlin Syndrome

Nevoid basal cell carcinoma syndrome, or Gorlin syndrome, is a rare autosomal dominant disorder characterized by multiple basal cell carcinomas, developmental defects including bifid ribs and other spine and rib abnormalities, palmar and plantar pits, odontogenic keratocysts, and generalized overgrowth.[65] The sonic hedgehog (SHH) signaling pathway directs embryonic development of a spectrum of organisms. Gorlin syndrome appears to be caused by germline mutations of the tumor suppressor gene *PTCH*, a *receptor* for SHH. Medulloblastoma develops in approximately 5% of patients with Gorlin syndrome. Furthermore, approximately 10% of patients diagnosed with medulloblastoma by the age of 2 years are found to have other phenotypic features consistent with Gorlin syndrome and harbor germline *PTCH* mutations.[66] Although Gorlin syndrome develops in individuals with germline mutations of *PTCH*, a subset of children with medulloblastoma harbor germline mutations of another gene, *SUFU*, in the SHH pathway, with accompanying LOH in the tumors.

Multiple Endocrine Neoplasia

The multiple endocrine neoplasia (MEN) disorders comprise at least three different diseases—MEN type 1, MEN type 2A, and MEN type 2B, which are all cancer predisposition syndromes that affect different endocrine organs. The most common features of MEN type 1 are parathyroid adenomas (about 90% of cases), pancreatic islet cell tumors (50% to 75% of cases), and pituitary adenomas (25% to 65% of cases).[67] MEN2A is associated with medullary thyroid carcinoma, parathyroid adenoma, and pheochromocytoma. MEN2B is a related disorder, but with onset of the tumors in early infancy, ganglioneuroma of the gastrointestinal tract, and skeletal abnormalities.

Although MEN1 is caused by mutation in the tumor-suppressor gene, MEN1, MEN2A, and MEN2B are caused by mutations in the proto-oncogene *RET*. Further studies

Table 27-5 Beckwith-Wiedemann Syndrome Genetic and Epigenetic Subgroups

	DNA	RNA	Karyotype	Frequency	Inheritance
A. Regional	Paternal 11p15 UPD		Normal lipis	10%-20%	Sporadic
	Disruption of *KCNQ10T1*		Duplication 11p15	1%	Sporadic
			Transl/Inver	1%	Sporadic
B. Domain 1	*H19* hypermethylation	*IGF2* LOI	Normal	2%	Sporadic
	Normal *H19* methylation	*IGF2* LOI	Normal	25%-50%	Sporadic
C. Domain 2	*CDKN1C* mutation	KNQ1OT1 LOI	Normal	5%-10%	Sporadic
	CDKN1C mutation		Normal	25%	AD
	KvDMR1 LOM		Normal	50%	Sporadic
D. Other	Unknown	Unknown	Normal	5%	AD
	Unknown		Normal	10%-20%	Sporadic

AD, Autosomal dominant; *LOI*, loss of imprinting; *LOM*, loss of methylation; *UPD*, uniparental disomy.

confirmed constitutional mutations in the *RET* proto-oncogene in families with MEN2A and 2B. The pattern of mutations seen in MEN2 families does not follow the "two-hit hypothesis" for tumor suppressor genes: the RET proto-oncogene is not inactivated, and there is no loss of the second allele in the tumors. Thus the predisposition to cancer in families with MEN2 is based on the inheritance of an activating mutation in the *RET* proto-oncogene. Genetic testing is possible by direct mutation analysis of the 10 exons of the gene.

DICER1 Syndrome

DICER1 syndrome is a very recently characterized phenotypic association of distinctive dysontogenic hyperplastic or overtly malignant tumors. The most frequent of these is the rare childhood lung malignancy pleuropulmonary blastoma. A wide spectrum of other, primarily endocrine, manifestations are evident: ovarian Sertoli-Leydig cell tumors (SLCT), nodular thyroid hyperplasia, pituitary blastoma, papillary and follicular thyroid carcinoma, cervical rhabdomyosarcoma, cystic nephroma, and possibly Wilms tumor.[68] Germline mutations in DICER1 have been identified in children and young adults affected with one or several of these tumors, and somatic DICER1 mutations have been variously identified in sporadic component tumors of the disorder. DICER1 is an endoribonuclease that processes hairpin precursor microRNAs (mi RNAs) into short, functional miRNAs. Mature 5′ miRNAs as well as other components of the RNA-induced silencing complex (RISC) downregulate targeted mRNAs.[69] Unlike the classical "two-hit" mechanism associated with inactivation of tumor suppressor genes, the effect of DICER1 loss of function appears to primarily result from an initial inactivating mutation that reduces by half the amount of wild-type DICER1 protein, whereas the second hit specifically knocks out production of 5′ mature miRNAs. Furthermore, penetrance of the mutations is highly variable, and the explanation for the wide spectrum of both hyperplastic and malignant neoplasms is not clear. Although many of the lesions in *DICER1*-mutation carriers are relatively indolent or benign, the risk in childhood of some potentially lethal tumors such as pleuropulmonary blastoma and pineoblastoma indicates a need for clinical surveillance particularly targeting the lungs, abdomen, and brain.

Molecular and Clinical Surveillance for Cancer Predisposition in Children

As in tumors of adults, continued investigation of the molecular alterations that underlie tumor pathogenesis in children can be expected to provide insights that will lead to a highly specific molecular therapeutic target and that in turn should lead to more specific, more efficacious, and less toxic therapies. Because tumors associated with highly penetrant genetic predispositions often occur early in life, such insights into pathogenesis also provide novel opportunities for the diagnosis of cancer in children. The evidence to justify the use and continued refinement of molecular analysis for tumor diagnosis, prognosis, and development of novel therapeutic avenues for children with cancer is overwhelming. The use of molecular screening as a tool for identification of children at risk for the purpose of developing rational clinical surveillance guidelines is more controversial. Several issues are worth noting. Based on recommendations of the American Society of Clinical Oncology,[70] genetic testing should only be undertaken with fully informed consent, including elements of risk assessment, psychological implications of test results (both benefits and risks), risks of employer or insurance discrimination, confidentiality issues, and options and limitations of medical surveillance or prevention strategies. When children are not competent to give informed consent, the main consideration should be for the welfare of the child. Although screening for some mutations, such as *TP53*, *RET*, or *RB*, is associated with clearly defined beneficial medical management decisions that lead to improved outcomes, it has been argued that presymptomatic identification of other gene mutations, such as in DICER1 syndrome, are of less obvious clinical benefit. Regardless of the scenario, the complexity of the issues underlies the importance that referral of these patients and families is made to an experienced multidisciplinary team including oncologists, geneticists, psychologists, and genetic counselors to facilitate the most appropriate management.

Key References

2. Knapke S, Nagarajan R, Correll J, et al. Hereditary cancer risk assessment in a pediatric cancer followup clinic. *Pediatric Blood Cancer*. 2012;58:85-89.
9. Rushlow DE, Mol BM, Kennett JY, et al. Characterisation of retinoblastoma without RB1 mutations: genomic, gene expression, and clinical studies. *Lancet Oncol*. 2013; pii: S1470-2045(13) 0045–7.
10. Brennan RC, Federico S, Bradley C, et al. Targeting the p53 pathway in retinoblastoma with subconjunctival Nutlin-3a. *Cancer Res*. 2011;71:4205-4213.
16. Rivera MN, Kim WJ, Wells J, et al. An X chromosome gene, WTX, is commonly mutated in Wilms tumor. *Science*. 2007;315:642-645.
21. Ohali A, Avigad S, Ash S, et al. Telomere length is a prognostic factor in neuroblastoma. *Cancer*. 2006;107:1391-1399.
22. Castel-Branco P, Zhang C, Lipman T, et al. Selective targeting of cancer stem cells by telomerase inhibition. *Clin Cancer Res*. 2011;17:111-121.
23. Trochet D, Bourdeaut F, Janoueix-Lerosey I, et al. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. *Am J Human Genet*. 2004;74:761-764.
24. Mossé YP, Laudenslager M, Longo L, et al. Identification of ALK as a major neuroblastoma susceptibility gene. *Nature*. 2008;455:930-935.

25. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45:279-284.
28. Erkizan HV, Kong Y, Merchant M, et al. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. *Nat Med*. 2009;15:750-756.
29. O'Neill A, Shah N, Zitomersky N, et al. Insulin-like growth factor 1 receptor as a therapeutic target in Ewing sarcoma: lack of consistent upregulation or recurrent mutation and a review of the clinical trial literature. *Sarcoma*. 2013; doi: 10.1155/2013/450478.
31. Pierron G, Tirode F, Lucchesi C, et al. A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion. *Nat Genet*. 2012;44:461-466.
32. Wexler LC, Helman LJ. Rhabdomyosarcoma and the undifferentiated sarcomas. In: Pizzo PP, ed. *Principles and Practice of Pediatric Oncology*. 4th ed. New York, NY: Lippincott Williams & Wilkins; 2006.
35. Williamson D, Missiaglia E, de Reyniès A, et al. Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma. *J Clin Oncol*. 2010;28:2151-2158.
38. Gripp KW, Lin AE. Costellow syndrome: a Ras/mitogen activated protein kinase pathway syndrome (rasopathy) resulting from HRAS germline mutations. *Genet Med*. 2012;14:285-292.
40. Pasic I, Shlien A, Durbin AD, et al. Recurrent focal copy-number changes and loss of heterozygosity implicate two noncoding RNAs and one tumor suppressor gene at chromosome 3q13.31 in osteosarcoma. *Cancer Res*. 2010;70: 160-171.
42. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 144: 27-40.
44. Lockwood WW, Stack D, Morris T, et al. Cyclin E1 is amplified and overexpressed in osteosarcoma. *J Med Design*. 2011;13:289-296.
46. Tinat J, Bougeard G, Baert-Desurmont S, et al. 2009 version of the Chompret criteria for Li-Fraumeni syndrome. *J Clin Oncol*. 2009;27:e108-e109.
49. Bougeard G, Baert-Desermont S, Tournier I, et al. Impact of the MDM2 SNP 309 and p53 Arg72Pro polymorphism on age of onset in Li-Fraumeni syndrome. *J Med Genet*. 2006;43:531-533.
50. Rausch T, Jones DT, Zapatka M, et al. Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. *Cell*. 2012;148:59-71.
51. Shlien A, Marshall CR, Pienkowska M, et al. Excessive genomic DNA copy number variation in the Li-Fraumeni cancer predisposition syndrome. *Proc Natl Acad Sci U S A*. 2008;32:11264-11269.
52. Marcel V, Palmero EI, Falagan-Lotsch P, et al. TP53 PIN3 and SNP 309 polymorphisms as genetic modifiers in the Li-Fraumeni syndrome: impact on age at first diagnosis. *J Med Genet*. 2009;46:766-772.
53. Masciari S, Van den Abeele, Diller LR, et al. F18-fluorodeoxyglucose-positron emission tomography/computed tomography screening in Li-Fraumeni syndrome. *JAMA*. 2008;299:1315-1319.
54. Villani A, Tabori U, Schiffman J, et al. Imaging and biochemical surveillance in TP53 mutation carriers with Li-Fraumeni syndrome: a prospective observational study. *Lancet Oncol*. 2011;12:559-567.
55. Giminez-Roqueplo AP, Dahia PL, Robledo M. An update on the genetics of paraganglioma, pheochromocytoma and associated hereditary syndromes. *Hormon Metab Res*. 2012;44:328-333.
56. Benn DE, Giminez-Roqueplo AP, Reilly JR, et al. Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. *J Clin Endocrinol Metab*. 2006;91:827-836.
57. Gimenez-Roqueplo AP, Tischler AS. Pheochromocytoma and paraganglioma: progress on all fronts. *Endocr Pathol*. 2012;23:1-3.
58. Ayala-Ramirez M, Chougnet CN, Habra MA, et al. Treatment with sunitinib for patients with progressive metastatic pheochromocytomas and sympathetic paragangliomas. *J Clin Endocrinol Metab*. 2012;97:4040-4050.
59. Pasini B, Stratakis CA. SDH mutations in tumorigenesis and inherited endocrine tumors: lesson from the pheochromocytoma-paraganglioma syndromes. *J Intern Med*. 2009;266:19-42.
60. Hao HX, Khalimonchuk O, Shraders M, et al. SDH5, a gene required for flavination of succinate dehydrogenase is mutated in paraganglioma. *Science*. 2009;325:1139-1142.
61. Eisenhofer G, Lenders JW, Timmers H, et al. Measurements of plasma methoxytyramine, normetanephrine, and metanephrine as discriminators of different hereditary forms of pheochromocytoma. *Clin Chem*. 2011;57:411-420.
62. Gimenez-Roqueplo AP, Caumont-Prim A, Houzard C, et al. Imaging work-up for screening of paraganglioma and pheochromocytoma in SDHx mutation carriers: a multicenter prospective study from the PGL. EVA Investigators. *J Clin Endocrinol Metab*. 2013;98:E162-E173.
68. Choong SC, Priest JR, Foulkes WD. Exploring the endocrine manifestations of DICER1 mutations. *Trends Mol Med*. 2012;18:503-505.
69. Bahubeshi A, Tischkowitz M, Foulkes WD. miRNA processing and human cancer: DICER1 cuts the mustard. *Sci Transl Med*. 2011;3:111ps46.

This page intentionally left blank

References

1. Landis SH, Murray T, Bolden S, et al. Cancer statistics. *CA Cancer J Clin*. 1998;1998(48):6-29.
2. Knapke S, Nagarajan R, Correll J, et al. Hereditary cancer risk assessment in a pediatric cancer followup clinic. *Pediatric Blood Cancer*. 2012;58:85-89.
3. Devesa SS. The incidence of retinoblastoma. *Am J Ophthalmol*. 1975;80: 263-265.
4. Knudson Jr AG, Hethcote HW, Brown BW. Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma. *Proc Natl Acad Sci U S A*. 1975;72:5116-5120.
5. Ellsworth RM. The practical management of retinoblastoma. *Trans Am Ophthalmol Soc*. 1969;67:462-534.
6. Squire J, Dryja TP, Dunn J, et al. Cloning of the esterase D gene: a polymorphic gene probe closely linked to the retinoblastoma locus on chromosome 13. *Proc Natl Acad Sci U S A*. 1986;83:6573-6577.
7. Cavenee WK, Dryja TP, Phillips RA, et al. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*. 1983;305:779-784.
8. Friend SH, Bernards R, Rogelj S, et al. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*. 1986;323:643-646.
9. Rushlow DE, Mol BM, Kennett JY, et al. Characterisation of retinoblastoma without RB1 mutations: genomic, gene expression, and clinical studies. *Lancet Oncol*. 2013; pii: S1470-2045(13) 0045–7.
10. Brennan RC, Federico S, Bradley C, et al. Targeting the p53 pathway in retinoblastoma with subconjunctival Nutlin-3a. *Cancer Res*. 2011;71:4205-4213.
11. Beckwith JB, Kiviat NB, Bonadio JF. Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms tumor. *Pediatr Pathol*. 1990;10:1-36.
12. Riccardi VM, Sujansky E, Smith AC, Francke U. Chromosomal imbalance in the aniridia-Wilms tumor association: 11p interstitial deletion. *Pediatrics*. 1978;61:604-610.
13. Mueller RF. The Denys-Drash syndrome. *J Med Genet*. 1994;31:471-477.
14. Sotelo-Avila C, Gonzalez-Crussi F, Fowler JW. Complete and incomplete forms of Beckwith-Wiedemann syndrome: their oncogenic potential. *J Pediatr*. 1980;96:47-50.
15. Ping AJ, Reeve AE, Law DJ, et al. Genetic linkage of Beckwith-Wiedemann syndrome to 11p15. *Am J Hum Genet*. 1989;44:720-723.
16. Rivera MN, Kim WJ, Wells J, et al. An X chromosome gene, WTX, is commonly mutated in Wilms tumor. *Science*. 2007;315:642-645.
17. Evans A, D'Angio G, Randolph J. A proposed staging system for children with neuroblastoma. *Cancer*. 1971;27:374-378.
18. Schwab M, Alitalo K, Klempnauer KH, et al. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature*. 1983;305:245-248.
19. Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature*. 1985;313:404-406.
20. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med*. 1999;341:1165-1173.
21. Ohali A, Avigad S, Ash S, et al. Telomere length is a prognostic factor in neuroblastoma. *Cancer*. 2006;107:1391-1399.
22. Castel-Branco P, Zhang C, Lipman T, et al. Selective targeting of cancer stem cells by telomerase inhibition. *Clin Cancer Res*. 2011;17:111-121.
23. Trochet D, Bourdeaut F, Janoueix-Lerosey I, et al. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. *Am J Human Genet*. 2004;74:761-764.
24. Mossé YP, Laudenslager M, Longo L, et al. Identification of ALK as a major neuroblastoma susceptibility gene. *Nature*. 2008;455:930-935.
25. Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. *Nat Genet*. 2013;45:279-284.
26. Whang PJ, Triche TJ, Knutsen T, et al. Cytogenetic characterization of selected small round cell tumors of childhood. *Cancer Genet Cytogenet*. 1986;21:185-208.
27. Karnieli E, Werner H, Rauscher Jr F, et al. The IGF-1 receptor gene promoter is a molecular target for the Ewing sarcoma-Wilms tumor 1 fusion protein. *J Biol Chem*. 1996;271:19304-19309.
28. Erkizan HV, Kong Y, Merchant M, et al. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. *Nat Med*. 2009;15:750-756.
29. O'Neill A, Shah N, Zitomersky N, et al. Insulin-like growth factor 1 receptor as a therapeutic target in Ewing sarcoma: lack of consistent upregulation or recurrent mutation and a review of the clinical trial literature. *Sarcoma*. 2013; doi: 10.1155/2013/450478.
30. Zoubek A, Dockhorn-Dworniczak B, Delattre O, et al. Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? *J Clin Oncol*. 1996;14:1245-1251.
31. Pierron G, Tirode F, Lucchesi C, et al. A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion. *Nat Genet*. 2012;44:461-466.
32. Wexler LC, Helman LJ. Rhabdomyosarcoma and the undifferentiated sarcomas. In: Pizzo PP, ed. *Principles and Practice of Pediatric Oncology*. 4th ed. New York, NY: Lippincott Williams & Wilkins; 2006.
33. Turc-Cuel C, Lizard-Nacol S, Justrabo E, et al. Consistent chromosomal translocation in alveolar rhabdomyosarcoma. *Cancer Genet Cytogenet*. 1986;19: 361-362.
34. Biegel JA, Meek RS, Parmiter AH, et al. Chromosomal translocation t(1;13) (p36;q14) in a case of rhabdomyosarcoma. *Genes Chromosome Cancer*. 1991;3:483-484.
35. Williamson D, Missiaglia E, de Reyniès A, et al. Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma. *J Clin Oncol*. 2010;28:2151-2158.
36. Scrable H, Witte D, Lampkin B, et al. Chromosomal localization of the human rhabdomyosarcoma locus by mitotic recombination mapping. *Nature*. 1987;329:645-647.
37. Scrable H, Cavenee W, Ghavimi F, et al. A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting. *Proc Natl Acad Sci U S A*. 1989;86:7480-7484.
38. Gripp KW, Lin AE. Costellow syndrome: a Ras/mitogen activated protein kinase pathway syndrome (rasopathy) resulting from HRAS germline mutations. *Genet Med*. 2012;14:285-292.
39. Mitelman F. Recurrent chromosome aberrations in cancer. *Mutat Res*. 2000;462:247-253.
40. Pasic I, Shlien A, Durbin AD, et al. Recurrent focal copy-number changes and loss of heterozygosity implicate two noncoding RNAs and one tumor suppressor gene at chromosome 3q13.31 in osteosarcoma. *Cancer Res*. 2010;70: 160-171.
41. Yamaguchi T, Toguchida J, Yamamuro T, et al. Allelotype analysis in osteosarcomas: frequent allele loss on 3q, 13q, 17p, and 18q. *Cancer Res*. 1992;52:2419-2423.
42. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 144: 27-40.
43. Worth LL, Lafleur EA, Jia SF, Kleinerman ES. Fas expression inversely correlates with metastatic potential in osteosarcoma cells. *Oncol Rep*. 2002;9:823-827.
44. Lockwood WW, Stack D, Morris T, et al. Cyclin E1 is amplified and overexpressed in osteosarcoma. *J Med Design*. 2011;13:289-296.
45. Li FP, Fraumeni Jr JF. Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. *J Natl Cancer Inst*. 1969;43:1365-1373.
46. Tinat J, Bougeard G, Baert-Desurmont S, et al. 2009 version of the Chompret criteria for Li-Fraumeni syndrome. *J Clin Oncol*. 2009;27:e108-e109.
47. Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*. 1990;250:1233-1238.
48. Srivastava S, Zou ZQ, Pirollo K, et al. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature*. 1990;348:747-749.
49. Bougeard G, Baert-Desermont S, Tournier I, et al. Impact of the MDM2 SNP 309 and p53 Arg72Pro polymorphism on age of onset in Li-Fraumeni syndrome. *J Med Genet*. 2006;43:531-533.
50. Rausch T, Jones DT, Zapatka M, et al. Genome sequencing of pediatric medulloblastoma links catastrophic DNA rearrangements with TP53 mutations. *Cell*. 2012;148:59-71.

51. Shlien A, Marshall CR, Pienkowska M, et al. Excessive genomic DNA copy number variation in the Li-Fraumeni cancer predisposition syndrome. *Proc Natl Acad Sci U S A*. 2008;32:11264-11269.
52. Marcel V, Palmero EI, Falagan-Lotsch P, et al. TP53 PIN3 and SNP 309 polymorphisms as genetic modifiers in the Li-Fraumeni syndrome: impact on age at first diagnosis. *J Med Genet*. 2009;46:766-772.
53. Masciari S, Van den Abeele, Diller LR, et al. F18-fluorodeoxyglucose-positron emission tomography/computed tomography screening in Li-Fraumeni syndrome. *JAMA*. 2008;299:1315-1319.
54. Villani A, Tabori U, Schiffman J, et al. Imaging and biochemical surveillance in TP53 mutation carriers with Li-Fraumeni syndrome: a prospective observational study. *Lancet Oncol*. 2011;12:559-567.
55. Giminez-Roqueplo AP, Dahia PL, Robledo M. An update on the genetics of paraganglioma, pheochromocytoma and associated hereditary syndromes. *Hormon Metab Res*. 2012;44:328-333.
56. Benn DE, Giminez-Roqueplo AP, Reilly JR, et al. Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. *J Clin Endocrinol Metab*. 2006;91:827-836.
57. Gimenez-Roqueplo AP, Tischler AS. Pheochromocytoma and paraganglioma: progress on all fronts. *Endocr Pathol*. 2012;23:1-3.
58. Ayala-Ramirez M, Chougnet CN, Habra MA, et al. Treatment with sunitinib for patients with progressive metastatic pheochromocytomas and sympathetic paragangliomas. *J Clin Endocrinol Metab*. 2012;97:4040-4050.
59. Pasini B, Stratakis CA. SDH mutations in tumorigenesis and inherited endocrine tumors: lesson from the pheochromocytoma-paraganglioma syndromes. *J Intern Med*. 2009;266:19-42.
60. Hao HX, Khalimonchuk O, Shraders M, et al. SDH5, a gene required for flavination of succinate dehydrogenase is mutated in paraganglioma. *Science*. 2009;325:1139-1142.
60a. Baysal BE, Ferrell RE, Willett-Brozick JE, et al. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science*. 2000; 287(5454):848-851..
60b. Niemann S, Muller U, Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nature Genet*. 2000; 26(3):268-270..
60c. Astuti D, Latif F, Dallol A, et al. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet*. 2001; 69(1):49-54..
60d. Burnichon N, Briere JJ, Libe R, et al. SDHA is a tumor suppressor gene causing paraganglioma. *Hum Mol Genet*. 2010; 19(15):3011-3020..
61. Eisenhofer G, Lenders JW, Timmers H, et al. Measurements of plasma methoxytyramine, normetanephrine, and metanephrine as discriminators of different hereditary forms of pheochromocytoma. *Clin Chem*. 2011;57: 411-420.
62. Gimenez-Roqueplo AP, Caumont-Prim A, Houzard C, et al. Imaging workup for screening of paraganglioma and pheochromocytoma in SDHx mutation carriers: a multicenter prospective study from the PGL. EVA Investigators. *J Clin Endocrinol Metab*. 2013;98:E162-E173.
63. Mannens M, Hoovers JM, Redeker E, et al. Parental imprinting of human chromosome region 11p15.3-pter involved in the Beckwith-Wiedemann syndrome and various human neoplasia. *Eur J Hum Genet*. 1994;2:3-23.
64. Reik W, Brown KW, Schneid H, et al. Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by altered imprinting pattern in the IGF2-H19 domain. *Hum Mol Genet*. 1995;4:2379-2385.
65. Gorlin RJ. Nevoid basal-cell carcinoma syndrome. *Medicine (Baltimore)*. 1987;66:98-113.
66. Cowan R, Hoban P, Kelsey A, et al. The gene for the naevoid basal cell carcinoma syndrome acts as a tumour-suppressor gene in medulloblastoma. *Br J Cancer*. 1997;76:141-145.
67. Chandrasekharappa SC, Guru SC, Manickam P, et al. Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science*. 1997;276: 404-407.
68. Choong SC, Priest JR, Foulkes WD. Exploring the endocrine manifestations of DICER1 mutations. *Trends Mol Med*. 2012;18:503-505.
69. Bahubeshi A, Tischkowitz M, Foulkes WD. miRNA processing and human cancer: DICER1 cuts the mustard. *Sci Transl Med*. 2011;3:111ps46.
70. Statement of the American Society of Clinical Oncology: genetic testing for cancer susceptibility. Adopted on February 20, 1996. *J Clin Oncol*. 1996;14: 1730-1736.

Sean M. Post, Hagop Kantarjian, and Alfonso Quintás-Cardama

28

Biology of Adult Myelocytic Leukemia and Myelodysplasia

Introduction

The genesis of human myeloid leukemia involves the deregulation of the differentiation and maturation programs of the hematopoietic myeloid lineage that originates from primitive stem cells with multilineage potential. Myeloid leukemias have been linked to the acquisition of chromosomal aberrations and/or somatic mutations that result in subversion of the proliferation, differentiation, and survival cellular programs. Historically, the different acute myeloid leukemia (AML) phenotypes have been classified into subtypes according to the specific myeloid lineage from which they morphologically appeared to arise. However, it is now widely recognized that the complexity of molecular genotypes largely exceeds the number of recognizable morphologic phenotypes. As more genetic alterations are being recognized in AML, most modern taxonomic efforts have dynamically integrated the wealth of available genetic and emerging molecular information with clinical correlates. Myelodysplastic syndromes (MDSs), on the other hand, are a collection of clonal malignancies arising in hematopoietic stem cells characterized by abnormalities in cellular differentiation, dysplastic changes, increased apoptosis, and a propensity to progress to AML. There is considerable overlapping regarding the types of cytogenetic abnormalities and gene mutations observed in AML and MDS. Most patients with AML or MDS still succumb to their malignancies, which underscores the need to develop novel therapeutic approaches.

Acute Myeloid Leukemia

Cytogenetic aberrations are detected in approximately 50% of patients with AML at the time of diagnosis and remain the strongest predictor of survival (Table 28-1). The remainder, however, will present with a normal karyotype. These patients, although traditionally classified as having intermediate-risk AML, exhibit very variable clinical outcomes, which suggests the involvement of other molecular events in the pathogenesis of this AML subtype. Patients whose pretreatment karyotype is abnormal frequently present with chromosomal losses and/or with balanced reciprocal translocations involving the fusion of a transcription factor important in normal hematopoiesis such as core binding factor (CBF), retinoic acid receptor alpha (RARa), or homeobox (HOX) family members. Patients with normal karyotypes frequently present with mutations in genes involved in survival, proliferation, and differentiation, such as the receptor tyrosine kinase receptors FMS-like tyrosine kinase 3 *(FLT3)* or nucleophosmin member 1 *(NPM1)*. The discovery of these mutations has remarkably improved current risk stratification algorithms in AML.

According to the structural chromosome aberrancies detected at diagnosis, patients can be classified in three risk categories, favorable, intermediate, and adverse. Patients with t(15;17)(q22;q12-21) have an excellent prognosis and those with t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22), that is, those with CBF AML, have a relatively favorable prognosis. By contrast, patients with inv(3)(q21q26)/t(3;3)(q21;q26), –7 or a complex karyotype (i.e., at least three chromosome aberrations) have a poor clinical outcome. The presence of the poor-risk cytogenetic abnormalities –5, del(7q), –17/17p–, –18, or –20, are frequently associated with complex karyotypes. The frequency of the latter is higher in patients with secondary AML, and it increases with the patient's age. The value of the pretreatment cytogenetic analysis has been validated across different age groups. Multiple studies in recent years have highlighted the prognostic importance of a number of gene mutations in patients with CN-AML (e.g., *NPM1*, *FLT3*, and *DNMT3a* mutations).

Table 28-1 Frequent Chromosomal Aberrations in AML

	Genes	Morphological Association	Incidence*
Translocations/Inversions			
t(8;21)(q22;q22)	*RUNX1;RUNX1T1*	M2 with Auer rods	6%
inv(16)(p13q22)or t(16;16)(p13;q22)	*CBFB;MYH11*	M4Eo	7%
t(15;17)(q22;q11-21)	*PML;RARA*	M3/M3V	7%
t(9;11)(p22;q23)	*MLL;AF9*	M5	2%
t(6;11)(q27;q23)	*MLL;AF6*	M4 and M5	~1%
inv(3)(q21q26) or t(3;3)(q21;q26)	*EVI;RPN1*	M1, M4, M6, M7?	~1%
t(6;9)(p23;q34)	*DEK;NUP214*	M2, M4	~1%
Chromosomal Imbalances			
+8	..	M2, M4, and M5	9%
−7/7q−	..	No FAB preference	7%
−5/5q−	..	No FAB preference	7%
−17/17p−	TP53	No FAB preference	5%
−20/20q−	..	No FAB preference	3%
9q−	..	No FAB preference	3%
+22	..	M4, M4Eo	3%
+21	..	No FAB preference	2%
+13	..	Mo, M1	2%
+11	MLL1†	M1, M2	2%
Complex karyotype‡			10%
Normal karyotype			44%

AML, Acute myeloid leukemia.
Source: Adapted from Grimwade D, Mrozek K. Hematology/Oncology. Clinics of North America. 2011, 25: 1135-1161.
*Determined among 1311 patients with de novo AML enrolled onto Study 8461 of the Cancer and Leukemia Group B.
†Partial tandem duplication of the *MLL1*.
‡Three or more chromosomal aberrations in the absence of t(8;21), inv(16)/t(16;16), t(15;17), or t(9;11).

Molecular Pathogenesis of AML

The most frequently mutated genes in AML encode transcription factors, which are typically implicated in chromosomal translocations that result in their inappropriate activation in the hematopoietic compartment, resulting in impairment of maturation and differentiation of myeloid cells (e.g., CBF translocations and *RARα* and *MLL* gene rearrangements) (Figure 28-1). Frequent types of mutations are those that confer a proliferative and survival advantage to cells (i.e., activating mutations). An example of the latter include mutations in *KIT* or *FLT3* (Table 28-2). Mutations in AML are a concept in flux, both during the natural course of the disease and during the course of therapy (i.e., clonal evolution), as shown by whole-genome sequencing analyses performed in paired samples obtained at diagnosis and at relapse. Of note, in some cases, new mutations were found on relapse in the dominant clone in the primary leukemia sample, whereas in others, these newly acquired mutations occurred in small subclones of the founding clone, which resulted in expansion and clonal dominance in the relapse sample. Importantly, varying proportions of clonal cells from the founding clone persisted after chemotherapy in all cases.

Mutations Disrupting the Function of Transcription Factors in AML

Two different types of transcription factors play a major role in hematopoiesis and therefore in the pathogenesis of AML. The first group consists of master regulatory transcription factors, which, like AML1, are implicated in the development of all hematopoietic lineages. Impairment of the signaling stemming from these types of transcription factors results in complete hematopoietic failure. A second category of transcription factors is involved in the development of specific hematopoietic lineages. For instance, GATA-1 skews the development of hematopoietic progenitors toward the erythroid lineage, whereas C/EBPα promotes granulocytic differentiation.

PML-RARα Rearrangements

Translocations involving the *retinoic acid receptor (RAR)* locus on chromosome 17, such as t(15;17)(q22;q11), drive the pathogenesis of acute promyelocytic leukemia (APL), which comprises 10%-15% of all cases of adult AML. Such translocations produce the *PML-RARα* transcript that encodes a fusion protein containing most of the functional domains of RARα (including the *RAR* binding domain and the DNA binding domain) and the majority of the *PML* gene. The breakpoints within the *PML* gene cluster locate to three different regions referred to as *breakpoint cluster region* (bcr) 1, 2, and 3. In addition to t(15;17)(q22;q11), there are at least two other variant translocations involving *RARα* associated with the APL phenotype. These include t(11;17)(q23;q21) and t(5;17)(q35;q21), which lead to the fusion of the *RAR?* gene to the promyelocytic leukemia zinc finger *(PLZF)* and *NPM1* genes, respectively. Transgenic mice expressing *PML-RARα*, *NPM/RARα*, or *PLZF/RARα* under the control of a human *Cathepsin* G exhibit an APL phenotype after a variably long latency. Comparison of gene expression profiles set by *PML-RARα* and *PLZF-RARα* have demonstrated the inhibition of genes involved in DNA repair, repression of myeloid transcriptional regulators, and activation of the WNT/Catenin and Jagged/NOTCH pathways, which promote self-renewal of leukemic cells.

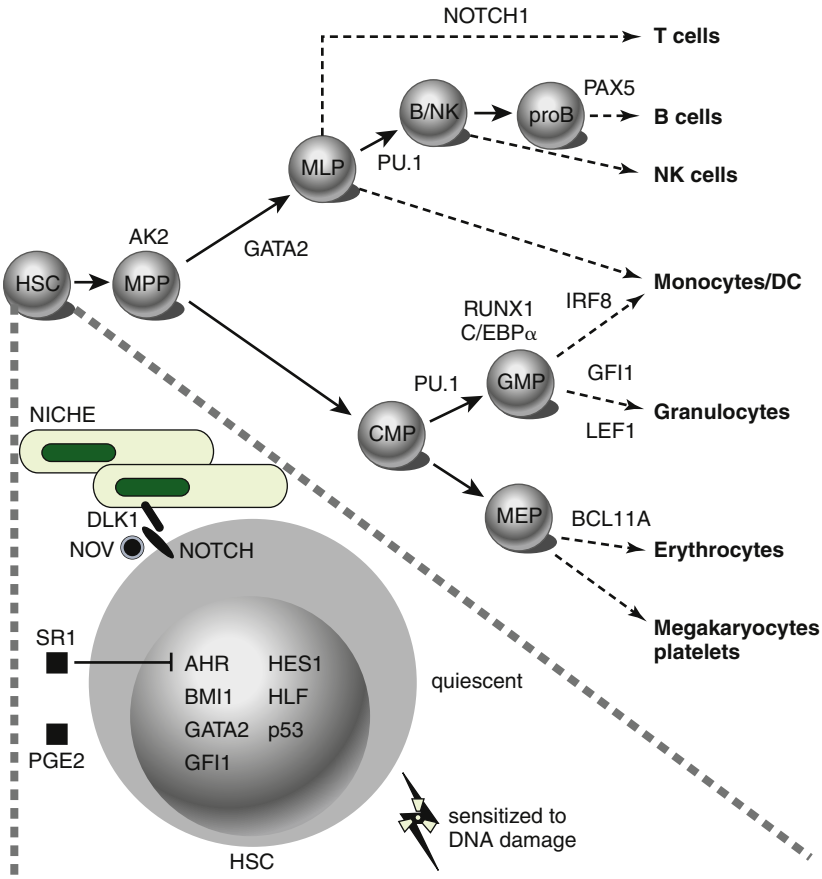


Figure 28-1 Transcription factors implicated in lineage specification of hematopoietic stem and progenitor cells. *From Doulatov S et al. Hematopoiesis: a human perspective.* Cell Stem Cell. *2012;10:120-136.*

Table 28-2 Association of Karyotypic Aberrations with Molecular Findings in AML

	Molecular Genetic Defect	Prevalence	Study
t(8;21)	KIT exon 8 mutation	2%	21
	KIT codon 816 mutation	11%	21, 25
	FLT3 ITD or D835 mutation	6%-11%	21, 25
inv(16)/t(16;16)*	KIT exon 8 mutation	24%-26%	21, 23
	KIT codon 816 mutation	7%-8%	21, 23
	FLT3 ITD or D835 mutation	8% 18%-26%	21, 23 23, 28
	NRAS mutation	9%-17%	23, 28
	KRAS mutation		
Normal karyotype	FLT3 ITD	28%-34%	73-76
	FLT3 TKD mutation	11%-14%	74-75
	NPM1 mutation	48%-64%	79-83
	CEBPA mutation	15%-18%	87, 99
	MLL1 PTD	8%-11%	89, 90
	NRAS mutation	14%	28
	KRAS mutation	4%	28
Gene overexpression	BAALC	..	98
t(6;9)	FLT3 ITD	90%†	75
+11	MLL PTD	91%†	94
+21	RUNX1 mutation	38%†	92
del (9q)	CEBPA mutation	41%	93

Source: Adapted from Estey et al. Acute Myeloid Leukaemia. *Lancet* 2006;368:1894-1907, with permission.
AML, Acute myeloid leukemia.
*≤70% of inv(16) leukemias have mutations in receptor tyrosine kinase or *RAS* genes.
†Prevalence based on a limited number of cases.

Patients with APL harboring *PLZF-RARα* fail to respond to *all-trans retinoic acid* (ATRA), although, paradoxically, both *PML-RARα* and *PLZF-RARα* contain identical *RAR* sequences and inhibit ATRA-induced gene transcription as well as cell differentiation.

The PML-RARα oncoprotein disrupts the interaction of retinoic acid and *RARα*, which converts the latter into a transcription activator, resulting in maturation arrest of hematopoietic progenitors at the promyelocyte stage. *PML-RARα* expression also disrupts PML localization, causing it to relocalize from discrete nuclear structures, the PML nuclear bodies, into microspeckled aberrant structures. PML-RARα acts as a dominant negative inhibitor of the PML protein, as well as the major heterodimeric partner of RARα, RXRα (retinoid X receptor). PML-RARα recruits several co-repressors, including the nuclear co-repressor (N-CoR). N-CoR inhibits transactivation from RARα target genes through the recruitment of the molecules sin3 and histone deacetylases, which in turn inhibit the binding of transcription factors and the binding of the transcriptional machinery to promoters, resulting in inhibition of gene expression for hematopoietic differentiation. Similarly, the PML moiety of the PML-RARα protein interacts with the DAXX co-repressor. Mutations preventing DAXX recruitment, although allowing PML-RARα dimerization,

abrogated the ability of PML-RARα to block hematopoietic differentiation and immortalize cells. ATRA is the mainstay of therapy in APL, inducing leukemic cell differentiation and remission in patients with t(15;17)/*PML-RARα* or t(5;17)/*NPM-RARα*. Similarly, arsenic trioxide (As_2O_3) has been demonstrated to be effective in the treatment of de novo as well as of ATRA-resistant t(15;17)/*PML-RARα* APL. Current evidence suggests that the combination of ATRA and As_2O_3 represents a valid alternative to chemotherapy-containing regimens for the treatment of APL.

Core Binding Factor AMLs

CBF is a heterodimeric transcription factor that consists of a DNA binding α-subunit, encoded by one of three members of the RUNX family (*RUNX1* or *AML1*, *RUNX2*, and *RUNX3*), and a β-subunit encoded by the *CBFβ* gene that increases DNA-binding affinity to the complex. Mutations involving CBF rearrangements occur in 15% of cases of AML and are associated with a favorable prognosis. CBF AML includes those carrying inv(16)/t(16;16), which gives rise to the fusion of *CBFβ* with the *smooth muscle myosin heavy-chain* gene (*MYH11* or *SMMHC*), and t(8;21), which is associated with the fusion transcript composed of the *AML1* and the *eight-twenty-one* (*ETO*) genes. Of note, in AML expressing the CBF translocation *AML1-ETO*, this fusion oncogene acts as a dominant negative inhibitor of native *AML1*. Similarly, the *CBFβ-MYH11* oncoprotein is a dominant negative inhibitor of CBF both in transactivation assays and during development. In knockin *CBFβ-MYH11* chimeric mice, *CBFβ-MYH11* expression alters adult multilineage hematopoietic differentiation. *CBFβ* also modulates the effect of *CBFβ-SMMHC* in adult hematopoiesis and leukemogenesis.

Rearrangements of the MLL and HOX Genes

Approximately 4% of patients with de novo AML have balanced translocations or insertions involving the *mixed-lineage leukemia* (MLL) gene. *MLL*-gene fusions are highly associated with previous therapy that includes topoisomerase-II inhibitors. The estimated incidence of this type of rearrangement in secondary treatment-related AML is 2% to 12%. *MLL* is the human homologue of *Drosophila TRX* and constitutes a maintenance factor for HOX proteins, which are central during development and hematopoiesis. The N terminus of *MLL*, which contains the AT-hook DNA-binding motif and a region homologous to DNA methyltransferase, is always retained in the fusion protein arising from chromosomal translocations, whereas the C terminus, which contains the activation and SET domains, is always replaced by the fusion partner. The *MLL* AT hooks bind to the minor groove of DNA, which facilitates the binding and recruitment of transcription factors to promoter elements.[1] *MLL* fusion genes can initiate both myeloid and lymphoid leukemogenic programs depending on the fusion partner, of which more than 65 have been thus far identified in AML. Differential activation of the Wnt/β-Catenin pathway is required for the maintenance of MLL leukemia stem cells, and MLL-AF9, one of the most frequent fusion transcripts, requires interacting with the Polycomb Group protein CBX8 to induce a leukemogenic transcriptional program. Approximately 5% to 10% of AML cases present with rearrangements of *MLL* consisting of an in-frame partial tandem duplication (*MLL*-PTD) of exons 11-5 or 12-5. *MLL*-PTD promotes increased histone H3/H4 acetylation and methylation of H3 Lys4 at *cis*-regulatory *HOXA* sequences. Mislocalized activity of the H3K79 histone methyltransferase DOT1L has been proposed as a driver of leukemogenesis in AML carrying MLL rearrangements. Pharmacological inhibition of DOT1L with the selective DOT1L inhibitor EPZ004777 selectively inhibits H3K79 methylation and blocks expression of leukemogenic genes with little effect on non–MLL-translocated cells, suggesting that DOT1L inhibition represents a potential therapeutic option for patients with MLL rearrangements.

HOX genes are frequently overexpressed in leukemia. Constitutive *HOX* gene activation is required for *MLL* fusion protein-mediated AML. Gene expression profiling analysis showed that the *HOXA4*, *HOXA9*, *HOXA10*, *PBX3*, and *MEIS1* homeobox genes are coexpressed across diverse cytogenetic groups but are undetectable in terminally differentiated hematopoietic cells. In AML, *HOX* genes are mainly disrupted via chromosomal translocation, such as the fusion of *NUP98*, *NUP214* (also known as *CAN*), or *MLL* to *HOXA9*, *HOXD13*, *DEK*, and *DDX10*. Overexpression of *HOXA6*, *HOXA7*, *HOXA9*, and the HOX cofactor *myeloid ecotropic viral integration site 1 (MEIS1)* has also been correlated with chromosome 11q23 abnormalities involving the MLL protein, which directly regulates the expression of *HOX* genes. The *caudal-type homeobox transcription factor 2 (CDX2)* is overexpressed in 90% of patients with AML in spite of its lack of expression in hematopoietic progenitors. *CDX2* overexpression in primary murine hematopoietic progenitors resulted in transplantable AML in vivo, which was associated with upregulation of *HOXB6* expression, a protein that is overexpressed in 40% of cases of CN-AML.[2] This suggests the possibility that *CDX2*-mediated deregulation of *HOX* genes is a major pathway to leukemogenesis.

Mutations in the C/EBPα and PU.1 Genes

The *C/EBPα* gene, which encodes the CCAAT/enhancer-binding-protein-alpha, is a member of the family of leucine-zipper (bZIP) transcription factors that couples lineage commitment to terminal differentiation and cell cycle arrest in the process of myeloid differentiation. *C/EBPα* initiates growth arrest through induction of *p21* and by disrupting

the E2F transcriptional complexes during the G1 phase of the cell cycle. Mutations in the *C/EBPα* gene occur in 15% to 19% of patients with AML and normal cytogenetics. *C/EBPα* mutations increase the capacity of bone marrow myeloid progenitors to proliferate and predispose mice to a granulocytic myeloproliferative disorder. In the absence of specific *C/EBPα* mutations, decreased expression may serve as an alternative mechanism that disrupts *C/EBPα* gene function. For example, *AML1-ETO* appears to indirectly suppress *C/EBPα* expression by inhibiting positive autoregulation of the *C/EBPα* promoter. Notably, *C/EBPα* mutations, when biallelic, are associated with a favorable prognosis in patients with cytogenetically normal (CN)-AML.

The transcription factor PU.1 is indispensable for myelomonocytic differentiation during normal hematopoiesis and for regulating the commitment of multipotent hematopoietic progenitors. The course of AML in mice after knockdown of *PU.1* includes a preleukemic stage during which immature myelomonocytic precursors accumulate in the bone marrow, followed by a leukemic phase with elevated leukemic blasts in peripheral blood. Also, PU.1-induced upregulation of *CSF1R* is crucial for leukemia stem cell potential induced by MOZ-TIF2. Despite its leukemogenic potential, *PU.1* mutations have been rarely found in human AML.

Mutations Altering Signal Transduction

Mutations at several oncogenes promoting cell growth have been shown to participate in the pathogenesis of AML. *FLT3* expression is restricted to $CD34^+$ cells and a subset of dendritic precursors, where it regulates proliferation, differentiation, and apoptosis.[3] Internal tandem duplication (ITD) within the *FLT3* juxtamembrane domain (exons 14 and 15) is among the most prevalent mutations in patients with CN-AML, being detected in about 30% of cases. Moreover, 7% of patients with AML harbor missense point mutations affecting the activation loop of the tyrosine kinase domain of FLT3 coded by exon 20, typically involving residue 835. There is evidence suggesting that *FLT3* mutations occur in leukemic stem cells. In fact, 84% of patients with AML carrying *FLT3-ITD* mutations exhibit the same mutation at relapse. *FLT3-ITD* mutations activate aberrant signaling, including STAT5, PI3K/AKT, and MAPK pathways, and repress *PU.1* and *C/EBPα*. *FLT3* gene mutations are associated with high relapse rates and poor prognosis. Small-molecule tyrosine kinase inhibitors directed against the constitutively activated FLT3 protein such as midostaurin, sorafenib, or quizartinib have shown encouraging results in clinical trials. Further development of these agents is being pursued in the context of chemotherapy or hypomethylation-based combinatorial approaches.

Activation of the KIT tyrosine kinase by somatic mutation has been documented in a variety of human malignancies, including core binding factor AML, clustering within exon 17 and exon 8. *KIT* mutations confer a higher risk of relapse in patients with CBF AML. Gain-of-function *KIT* mutations may serve as a target for tyrosine kinase inhibitors (e.g., dasatinib), and their activity warrants further investigation in CBF AML harboring such mutations.

RAS oncogenes encode a family of membrane-associated proteins that regulate proliferation, differentiation, and apoptosis. The RAS proteins oscillate between a guanosine triphosphate (GTP)- and a guanosine diphosphate (GDP)-bound state. GDP-bound RAS is incapable of activating signal transduction pathways. *NRAS* mutations can be detected in 10% of patients with AML. Mutated RAS proteins are constitutively activated, which is held in their GTP-bound status, and efficiently induce an AML-like disorder in a mouse bone marrow transplantation model. The prognostic impact of RAS mutations in AML remains controversial. MEK inhibitors are currently being tested in patients with AML carrying *RAS* mutations.

Other Genetic Events Implicated in the Pathogenesis of AML

Mutations of the Nucleophosmin Gene

The *NPM1* gene encodes a nucleus-cytoplasm shuttling protein implicated in preventing nucleolar protein aggregation, regulation of ribosomal protein assembly, initiation of centrosome duplication, and regulation of *p53*, *p19ARF*, and HDM2. *NPM1* is involved in the control of primitive hematopoiesis, and mice lacking *NPM1* alleles develop a syndrome reminiscent of human myelodysplasia. Translocations involving the *NPM* gene cause cytoplasmic dislocation of the NPM protein. *NPM1* mutations are found in approximately 35% to 40% of adult patients with primary AML and are associated with high sensitivity to cytarabine-based therapy and to higher complete remission rates. Most of the cases (60% to 80%) carrying mutant *NPM1* alleles correspond to patients with CN-AML. *NPM1* mutations are typically heterozygous and almost exclusively map to exon 12.[4-6] A duplication of a TCTG tetranucleotide at position 956 to 959 accounts for 75% to 80% of cases.[6,7] Notably, NPM1 mutations are associated with a very favorable prognosis in the presence of wild-type FLT3 alleles, particularly when associated with IDH1 mutations. The recent development of experimental mouse models lacking NPM1 alleles or carrying NMP1 mutations will aid in understanding the role of *NPM1* in

the pathogenesis of AML and in developing novel targeted agents.

Mutations in Genes Involved in Epigenetic Patterning and Chromatin Conformation

DNA methylation is a common mode of epigenetic regulation, typically involving cytosine residues that reside within GC-rich promoter regions called *CpG islands*. Another means of epigenetic regulation is via histone modifications. Histones, like DNA, can be methylated, but they are also acetylated, phosphorylated, sumoylated, and ubiquitinated. Histone modifications can result in either gene activation or repression. Mutations in a series of genes encoding proteins involved in epigenetic pathways are frequently found in patients with CN-AML, including *ASXL1* and *MLL*, which encode histone modifiers, and *DNMT3A*, *TET2*, and *IDH1/2*, which encode proteins that regulate cytosine modifications (Figure 28-2).

DNMT3A is an enzyme that catalyzes cytosine methylation, which is critical in DNA imprinting and modulation of gene expression. An analysis of 281 AML samples reported that 22% of cases carried mutations in *DNMT3A*, a frequency that increased to 34% among those with CN-AML. *DNMT3a* mutations were associated with a markedly shorter overall survival compared to that of patients carrying wild-type *DNMT3A* alleles (12.3 vs. 41.1 months).

Mutations in the *TET2* gene are found in 8% to 23% of patients with AML and have been associated with a poor prognosis (Figure 28-3). The role of TET2 mutations in the pathogenesis of AML is not well understood. TET proteins catalyze the conversion of 5-methylcytosine, which acts as a transcriptional repressor, to 5-hydroxymethylcytosine, thus potentially promoting transcription. The enzymatic activity of TET2 is inhibited by 2-hydroxyglutarate (2-HG), which is found at high levels in cells carrying mutant *IDH1/2* alleles. Mutations at the *IDH* alleles have been described at frequencies ranging from 15% to 20% overall and from 25% to 30% among those with CN-AML. The most frequent mutations in AML take place at the arginine 132 residue (R132) in *IDH1*, and the corresponding arginine 172 (R172) residue in *IDH2*, as well as at the arginine 140 (R140) residue in *IDH2*. Available data suggest that *IDH2* R140Q is associated with a favorable prognosis, whereas *IDH1* R132 confers a worse prognosis. *IDH2* R172 does not appear to affect the prognosis of patients with AML. Of note, *TET2* and

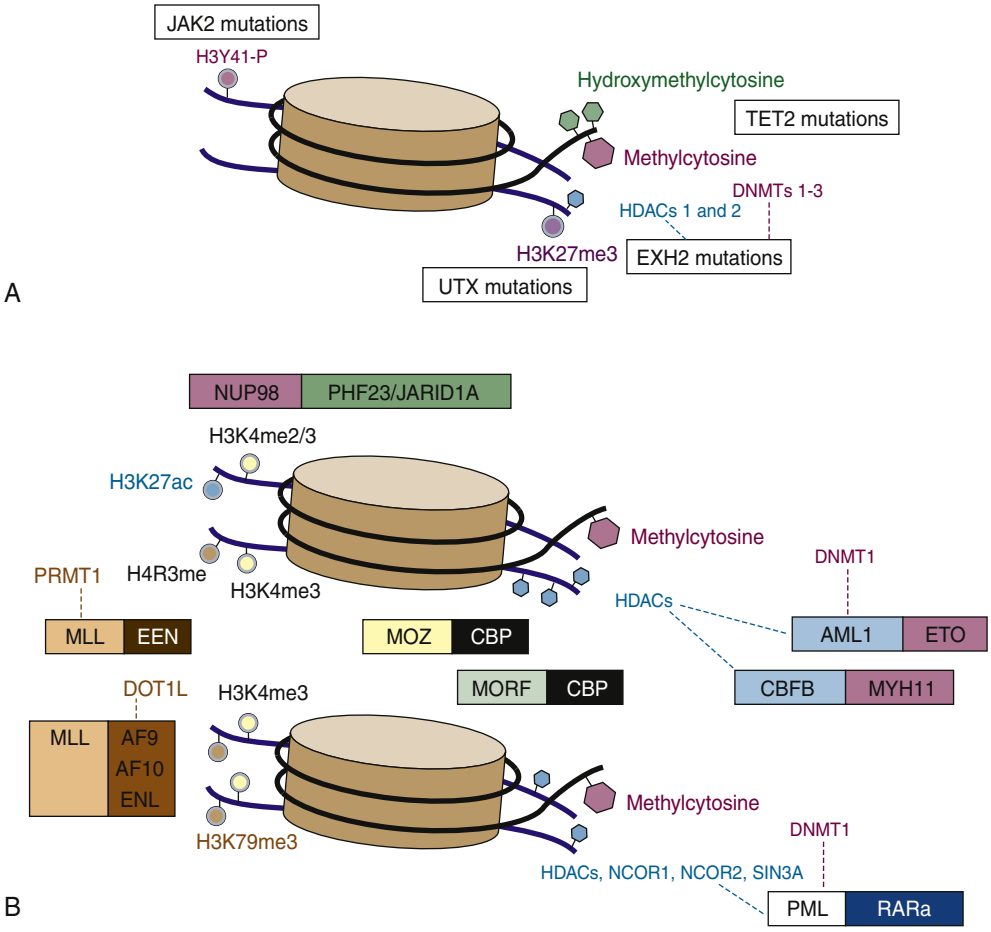
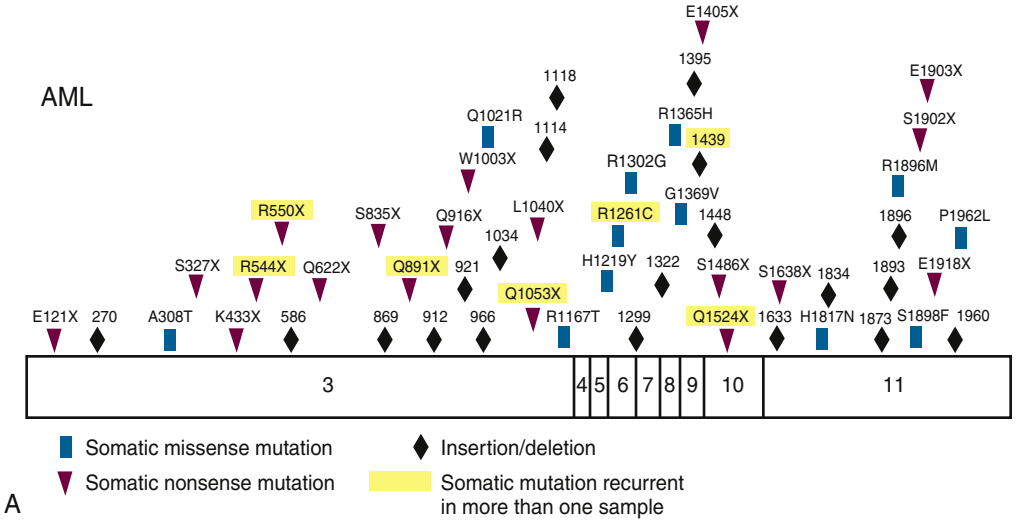


Figure 28-2 Simple mutations and chromosomal translocations in the epigenetic machinery in AML and MDS (A) Somatic mutations that affect the epigenetic machinery include gain-of-function mutations in JAK2 (which can phosphorylate histone 3 tyrosine 41) and loss-of-function mutations in UTX, EZH2, and TET2. **(B)** Epigenetic modifying genes can also be altered via chromosomal translocations such as those involving MLL fusions that lose H3K4 methyltransferase and gain H3K79 methyltransferase activity, JARID1A/PHF23 fusions that regulate H3K4 di/tri-methylation, and fusions such as PML-RARA or those involving core binding factors, which interact with histone deacetylases and modulate chromatin state. *From Abdel-Wahab O, Levine RL. EZH2 mutations: mutating the epigenetic machinery in myeloid malignancies.* Cancer Cell. *2010;18:105-107.*

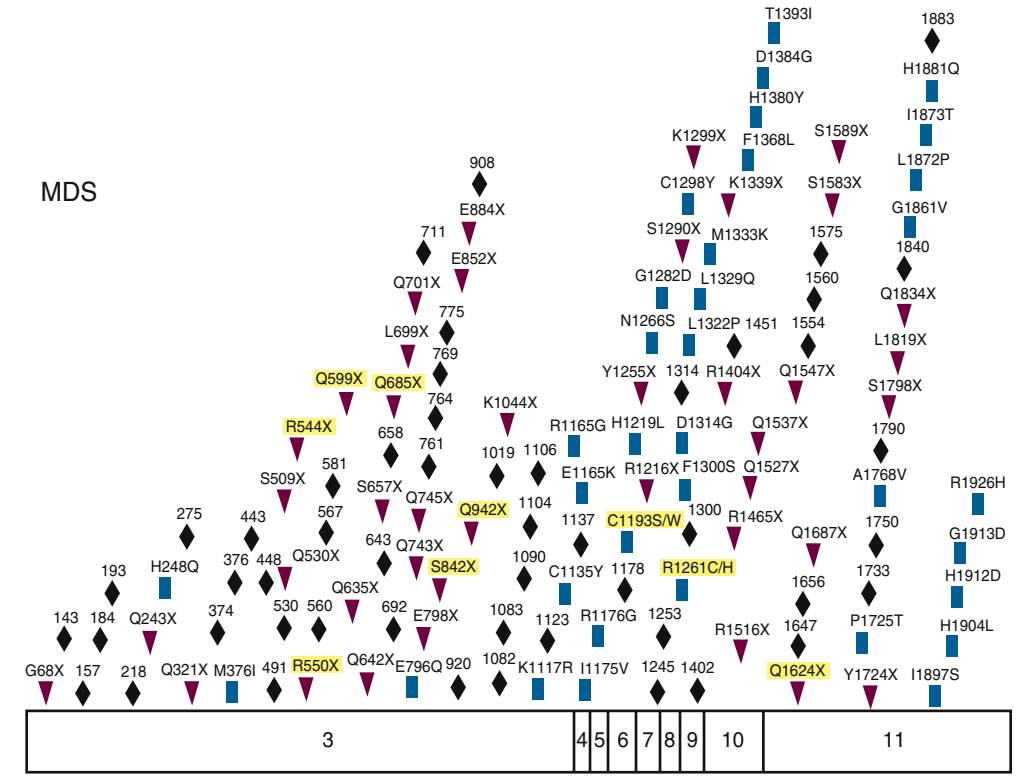
IDH mutations are mutually exclusive. *IDH* proteins convert isocitrate to α-KG, and *IDH* mutations result in neomorphic alleles that encode proteins that produce 2-HG in excess, which in turn inhibit the activity of *TET2*. The end result of both *TET2* and *IDH* mutations is the inability of cells to metabolize 5-methylcytosine to 5-hydroxymethylcytosine, thus inducing a hypermethylation phenotype. This suggests that possibility of using hypomethylating agents for the treatment of *TET2*- or *IDH*-mutated AML, although so far small trials have not shown any significant benefit. In addition, small molecules are being developed to inhibit the activity of mutated *IDH1/2*.

Overexpression of Specific Genes in AML

Overexpression of *BAALC*, the brain and acute leukemia gene, mRNA in the cytoplasm of peripheral blood blasts portends an adverse clinical outcome in terms of both failure to achieve complete response and shorter overall survival. The *MN1* gene is occasionally overexpressed in AML and is associated with worse overall survival. A similar phenomenon



A



B

Figure 28-3 Somatic acquired *TET2* mutations Patients with **(A)** AML or **(B)** MDS acquire somatic missense, nonsense, and frameshift mutations in *TET2*. *From Cimmino L, Abdel-Wahab O, Levine RL, et al. TET family proteins and their role in stem cell differentiation and transformation.* Cell Stem Cell. *2011;9:193-204.*

has been demonstrated among patients whose AML cells overexpress the v-ets erythroblastosis virus E26 oncogene homologue (*ERG*) gene, particularly among patients with low *BAALC* expression, as well as among those overexpressing the ecotropic viral integration site 1 (*EVI1*) gene. Elevated FOXO expression is present in 40% of cases of AML and is necessary to maintain leukemia-initiating cells. Resistance to FOXO depletion is mediated by JNK/c-JUN signaling. Over the past few years, a number of studies have correlated certain gene mutations with different patterns of microRNA (miR) expression. For instance, *NPM1* mutations associate with upregulation of miR-10a and miR-196a, whereas *FLT3* mutations associate with upregulation of miR-155 and *C/EBPα* with upregulation of miR-181 in CN-AML. Also, miR-10a, miR10b, and miR-196a-1 correlate with expression of *HOX* genes in CN-AML.

Myelodysplastic Syndromes

MDS is a heterogeneous group of clonal disorders of the hematopoietic stem cell, characterized by excessive apoptosis, maturation abnormalities of hematopoietic precursors manifested as dysplastic changes, and ineffective hematopoiesis. Unlike other malignant hematologic disorders, the biologic hallmark of the stem cell in MDS is a limited ability for self-renewal and differentiation. Furthermore, MDS has a tendency to transform into AML. Indeed, approximately 30% to 40% of cases evolve to AML. The survival of patients with MDS is quite heterogeneous, ranging from weeks to years. In an attempt to incorporate clinical features associated with prognosis in MDS, the International Prognostic Scoring System (IPSS) was developed. This system identified the presence of specific cytogenetic aberrancies, the percentage of blasts in the bone marrow, and the number of cytopenias as the most important variables in disease outcome.[8] Patients with therapy-related MDS are usually refractory to standard chemotherapy-based therapies, and their prognosis is very poor.

Although it is established that MDs arises from primitive hematopoietic progenitors, our understanding of the pathobiology that drives these diseases is incomplete. Some of the difficulties in determining the critical defects responsible for MDS can be explained by the fact that several programs such as cellular differentiation, apoptosis, and/or proliferation contribute to the etiology of this disease. MDS, like other cancers, results from multiple genetic alterations, likely acquired in a stepwise fashion, which frequently result in transformation to AML (Figure 28-4). This is in consonance with other models of molecular progression described in solid tumors such as colon or pancreatic cancer.

Cytogenetic Abnormalities in MDS

The main prognostic factors in patients with MDS are chromosomal abnormalities. An abnormal karyotype is present in approximately 50% of patients at diagnosis. Although multiple chromosomal lesions have been associated with MDS, losses of chromosomes 5 and/or 7 are associated with a worse prognosis among patients with de novo MDS. The precise genomic regions and genes responsible for the phenotypes observed in patients with specific chromosomal abnormalities are being revealed. Two commonly deleted regions (CDRs) have been defined: 5q33.1, which is associated with the 5q– syndrome, and 5q31, which is associated with therapy-related MDS and progression to AML. *RPS14* has been shown to be critical for the erythroid phenotype observed in the 5q– syndrome; miR-145 and miR-146 have been associated with an elevated platelet count. Approximately 10% of patients will present

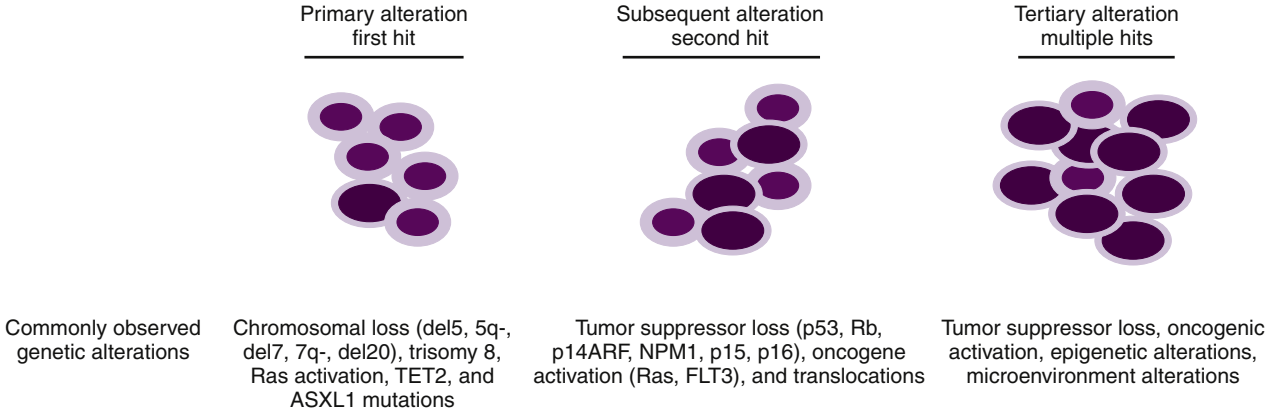


Figure 28-4 Progression of MDS to AML Primary somatic mutations in hematopoietic stem cells or myeloid progenitors result in dysplastic phenotypes. Subsequent genetic lesions that confer proliferation or anti-apoptotic advantages aid in the clonal evolution of the disease. Following numerous genetic anomalies, the dysplastic clone is rapidly amplified and becomes dominant.

with abnormalities of chromosome 7, where three CDRs have been identified. Importantly, EZH2, which acts as the catalytic component of the histone H3 lysine 27 methyltransferase polycomb repressive complex 2 (PRC2), maps to 7q36, which may explain why chromosome 7 losses are so frequent in MDS. Trisomy 8 occurs in 8% of patients and is also associated with poor prognosis in MDS. Other chromosomal abnormalities have been associated with a more favorable prognosis such as 20q− or −Y.

Loss of miRNAs 145/146

The miRNAs *miR-143*, *miR-145*, and *miR-146a* mapping at 5q33 are significantly reduced in bone marrow cells isolated from 5q− syndrome patients. *miR-145* and *miR-146a* regulate the toll-interleukin-1 receptor domain-containing adaptor protein *(TIRAP)* and tumor necrosis factor receptor–associated factor 6 *(TRAF6)*. TIRAP is a regulator of TRAF6, an E3 ubiquitin ligase, required for nuclear factor κB (NFκB) activation. Collectively, these proteins regulate innate immunity. Haploinsufficiency of *miR-145* and *miR-146a* resulted in increased *TIRAP* and *TRAF6* expression and activity in hematopoietic stem/progenitor cells. Transplantation of haploinsufficient *miRNA145/146a* cells or *TRAF6*-overexpressing cells into recipient mice resulted in several hallmark MDS/5q− syndrome phenotypes, such as thrombocytosis, neutropenia, dysplastic megakaryopoiesis, and propensity to transform to AML. These findings link innate immunity to MDS pathogenesis.

NUP98-HOX13 Translocation

NUP98 maps to 11p15.5 and can partner with multiple genes by chromosomal translocation in MDS. In t(2;11)(q31;p15), NUP98 fuses with HOXD11 or HOXD13.[9] Transgenic mice expressing NUP98-HOXD13 exhibit anemia, decreased neutrophil and lymphocyte counts, dysplastic changes, and increased bone marrow cellularity, thus recapitulating an MDS phenotype. Furthermore, a subset of *NUP98-HOX13* transgenic mice also transforms to AML after a latency of approximately 1 year, suggesting the need for secondary hits for transformation, which have been shown to consist of activating RAS mutations.

Gene Mutations in MDS

Mutations in the Spliceosome Machinery

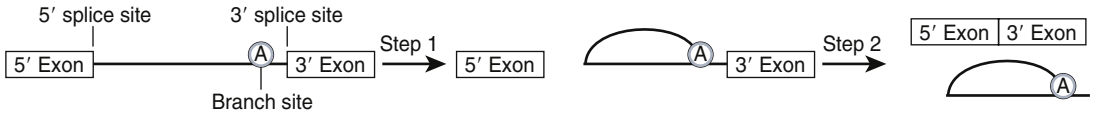
Spliceosomes are complexes composed of small nuclear RNA (snRNA) that remove introns in protein-encoding genes. Several groups, by means of next-generation sequencing platforms, have unveiled the presence of somatic mutations in genes encoding proteins involved in the spliceosome machinery in 45% to 85% of patients with MDS or chronic myelomonocytic leukemia (CMML). Collectively, eight different splicing genes—SF3B1, SRSF2, U2AF35 (also known as U2AF1), ZRSR2, SF3A1, PRP40B, SF1, and U2AF65—can be found mutated in MDS, constituting, as a group, the most frequently detected mutations in MDS (Figure 28-5). These splicing factors are highly expressed in hematopoietic lineages and are involved in recognition of the canonical 3′ DNA splice elements. Similar gene mutations can also be found at low frequencies in chronic lymphocytic leukemia (9% to 15%) or myeloproliferative neoplasms (3% to 9%), and in rare instances of solid cancers (e.g., breast or renal cancer). In MDS, these mutations are always present in the heterozygous state and are almost mutually exclusive. Across studies, mutations in SF3B1 have been described in 20% to 45% of patients and in 65% to 85% of those with MDS with ringed sideroblasts. It is hypothesized that altered splicing of key cancer genes might result in oncogenic gain or loss of function. In addition, genes such as SF3B1 might have oncogenic nonsplicing mechanisms because they have been shown to be implicated in cell cycle control and Hox gene regulation. The impact of these genes has not been clearly delineated, but SF3B1 mutations appear to confer a more favorable prognosis, whereas U2AF1 mutations are associated with poor prognosis among patients with MDS.

Gene Mutations Altering Epigenetic Regulation in MDS: TET2, IDH1/2, and ASXL1

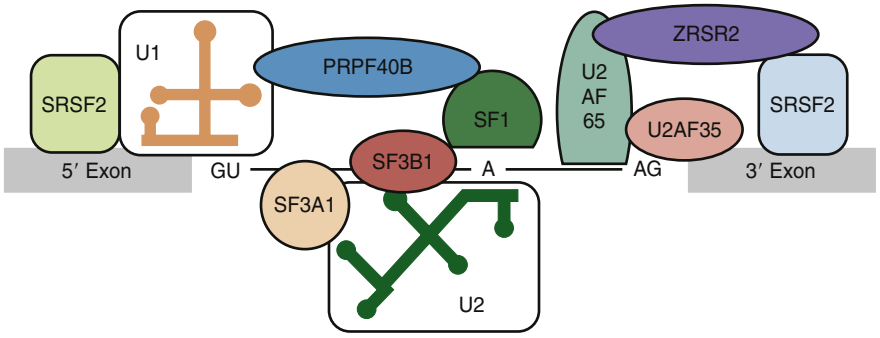
There is now growing evidence that in addition to genetic changes, epigenetic modifications play a critical role in the pathogenesis of MDS.[10,11] Loss-of-function mutations in *TET2*, a protein involved in global DNA demethylation, occurs in approximately 20% of patients with MDS.[12,13] However, TET2 mutations are not responsible for the dysplastic changes observed in the bone marrow of patients with MDS. The complete loss of *Tet2* resulted in myeloproliferation, increased repopulation of transplanted cells in recipient mice, and expansion of the hematopoietic progenitor pool. *Tet2*$^{-et}$ mice display phenotypes reminiscent of MDS with a short latency. The impact of *TET2* mutations on the outcome of patients with MDS remains controversial, with more recent reports indicating no impact on overall survival.

Much like in AML, *IDH1/2* mutations in MDS are heterozygous and appear to act as dominant negatives. However, IDH1/2 mutations are significantly less frequent in MDS than in AML, with frequencies ranging from 5% to 10%. A recent genetically engineered mouse model in which the *IDH1* R132H mutation was expressed in the hematopoietic system has been reported. Cells carrying the mutation exhibit high 2-HG levels, which are associated with an expansion of the stem cell pool and epigenetic changes frequently seen in MDS and AML (i.e., DNA and histone

A



B



C

Gene	Mutation Description	Mutational Frequency								
		RARS/ RCMD-RS	MDS without RS	CMML	t-MDS/ sAML	*De novo* AML	MPN	ALL	NHL	CLL
SF3B1	Predominant heterozygous missense mutations at K700, R625, and H662.	57%-75.3%	6%-20%	4.5%-5%	4.8%	2.6%-5%	3%-4%	nd	nd	5%
SRSF2	Recurrent heterozygous missense mutations at P95.	1.5%	11.6%	28.4%	6.5%	0.7%	1.9%	—	—	nd
U2AF35	Recurrent heterozygous missense mutations at S34 and Q157.	—	11.6%	8.0%	9.7%	1.3%	1.9%	—	—	nd
ZRSRS2	Missense, nonsense, and frameshift mutations throughout the open reading frame.	1.4%	7.7%	8.0%	1.6%	0.7%	1.9%	nd	nd	nd
SF3A1	Missense mutations throughout the open reading frame.	—	1.3%	1.1%	1.6%	0.7%	—	nd	nd	nd
PRPF40B	Missense mutations throughout the open reading frame.	—	1.9%	—	1.6%	0.7%	1.9%	nd	nd	nd
U2AF65	Missense mutations throughout the open reading frame.	—	0.6%	1.1%	—	—	—	nd	nd	nd
SF1	Missense mutations in the proline-rich C-terminal domain.	—	1.3%	—	—	—	1.9%	nd	nd	nd

Figure 28-5 Mutations in genes encoding spliceosomal proteins Five small ribonuclear proteins (snRNPs) and more than 50 accessory proteins complex to form the spliceosome at the exon/intron junction of pre-mRNA molecules. Following U1 snRNP and U2AF assembly, the U2 snRNP, the U4-6 tri-snRNP, and other splicing factors are assembled sequentially to form the spliceosome. SF_3B_1 and SF_3A_1 are components of U2 snRNP. SR proteins bind to an exonic-splicing enhancer region to directly recruit splicing machinery by interacting with $U2AF_{35}$ and ZRSR2. This interaction is critical in defining exon/intron boundaries. **(A)** Spliceosomal proteins found to be mutated in myeloid malignancies. **(B)** Frequency of mutations in spliceosomal proteins in different hematological malignancies including AML and MDS. *ALL,* Acute lymphoblastic leukemia; *CLL,* chronic lymphocytic leukemia; *CMML,* chronic myelomonocytic leukemia; *MPN,* myeloproliferative neoplasms; *NHL,* non-Hodgkin lymphoma; *RARS,* refractory anemia with ring sideroblasts; *RCMD-RS,* refractory cytopenia with multilineage dysplasia and ring sideroblasts; *sAML,* secondary AML; *t-MDS,* therapy-related MDS. A *dash* indicates that mutations have not been detected; *nd* indicates that sequencing was not done. ***A*** *and* ***B*** *from Padgett RA. New connections between splicing and human disease.* Trends Genet. *2012;28:147-154.* ***C*** *from Abdel-Wahab O, Levine RL. The spliceosome as an indicted conspirator in myeloid malignancies.* Cancer Cell. *2011;20:420-422.*

hypermethylation). Recent studies suggest that *IDH1* mutations may portend an adverse prognosis in MDS, whereas *IDH2* mutations had no impact.

ASXL1 (additional sex-comb like-1) encodes a member of the polycomb family of chromatin-binding proteins that, depending on the cellular context, either activates or silences genes. *ASXL1* alleles are mutated at the C terminus of the protein, and mutations can be detected in approximately 10% to 20% of patients with MDS and in 17% of those with AML. Loss of ASXL1 function results in aberrant repression or activation of numerous genes. Deletion of *Asxl1* in the germline of mice resulted in an embryonic lethal phenotype in some animals. Those surviving exhibited disrupted expression of the Homeobox family of *Hox* genes and defects in differentiation of lymphoid and myeloid progenitor cells but not in multipotent progenitors. The lack of an informative phenotype in the $Asxl1^{-/-}$ mice could be due in part to compensatory activities of the *ASXL1* homologues, *ASXL2* and *ASXL3*. However, Asxl1 functions are most frequently disrupted by DNA mutations that result in frameshift mutations. These types of mutations often confer dominant negative activities, which result in phenotypic changes quite different from those resulting from allelic loss. Knockin mice harboring *ASXL1* mutations are warranted

to fully examine the role of these mutations in the pathogenesis of MDS. *ASXL1* frameshift mutations have been associated with a reduced time to progression to AML and shorter survival.

RUNX1 Mutations

RUNX1 (also known as *AML1*) is a transcription core binding factor and represents the third most frequently mutated gene in MDS, being detected in 7% to 15% of cases. *Runx1* knockout mice die at the embryonic stage with no evidence of definitive hematopoiesis. When *Runx1* is deleted in adult hematopoietic tissues, mice expand their myeloid compartment and exhibit inefficient platelet production. Mutations in the Runt domain of Runx1 in mice are associated with a dominant negative/gain-of-function phenotype characterized by increased blast burden and AML-like disease.

NPM1 Mutations

NPM1 maps to chromosome 5 and is mutated in 5% of cases of MDS. NPM1 regulates the p53 pathway via $p14^{ARF}$. Genetic deletion of both *NPM1* alleles in mice results in embryonic lethality due to severe anemia. However, $Npm1^{+/-}$ mice are viable and present with several hematopoietic anomalies consistent with MDS, including abnormal platelet counts and erythroid or megakaryocytic dysplasia. In addition, aged $Npm1^{+/-}$ mice had an increased rate of incidence of AML. This model clearly establishes *NPM1* as a true tumor suppressor in MDS. *Npm1* mutant mice harboring a humanized *Npm1* mutation driven by a hematopoietic promoter have also been developed. These mice also exhibit myeloid-specific phenotypes, but they are more reminiscent of a myeloproliferative neoplasm.

TP53 Mutations

TP53 maps to 17p and is found mutated in 5% to 15% of patients with de novo MDS. *TP53* mutations portend a very poor prognosis regardless of IPSS score, and they are associated with advanced disease and complex karyotypes. *TP53* mutations to chromothripsis and complex DNA rearrangements in myeloid malignancies. TP53 activation has been demonstrated to be critical for the erythroid phenotype induced by *RPS14* haploinsufficiency in 5q- syndrome patients.

Alterations in the Bone Marrow Microenvironment

Although genetic and epigenetic factors clearly play a role in the pathogenesis of MDS and its progression to AML, little is known about the impact of alterations in the bone marrow niche. The importance of such signals has been recently demonstrated in one study that used Osterix-mediated Cre expression to delete the *Dicer* gene in osteoprogenitor cells.[14] Mice harboring *Dicer* deletions in osteoprogenitor cells exhibited MDS features such as cytopenias and morphologic changes in hematopoietic cells, demonstrating that bone marrow alterations may play an important role in MDS pathogenesis.

Concluding Remarks

The increasing sophistication of genome-wide sequencing techniques as well as of genetically engineered murine models has provided important insights into the pathogenesis of myeloid malignancies. These advances have also opened new avenues for the development of targeted therapies directed against mutant proteins present in AML or MDS cells, such as FLT3, KIT, IDH1/2, or RAS. However, a better understanding of the various cellular functions and signaling pathways subverted by these mutant proteins is warranted to extend the concept of targeted therapy in AML beyond the isolated paradigm that constitutes the success of ATRA and arsenic trioxide therapy in PML expressing *PML-RARα*. The alterations in transcription factor expression observed in AML or MDS are truly "tumor specific" and, as such, provide novel targets for therapy. However, designing therapeutic modalities aimed at modulating transcription factors remains challenging.

References

1. Dash A, Gilliland DG. Molecular genetics of acute myeloid leukaemia. *Best Pract Res Clin Haematol*. 2001;14:49-64.
2. Giampaolo A, Felli N, Diverio D, et al. Expression pattern of HOXB6 homeobox gene in myelomonocytic differentiation and acute myeloid leukemia. *Leukemia*. 2002;16:1293-1301.
3. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer*. 2003;3:650-665.
4. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352:254-266.
5. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood*. 2006;107:4011-4020.
6. Boissel N, Renneville A, Biggio V, et al. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. *Blood*. 2005;106:3618-3620.
7. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. *Blood*. 2007;109:874-885.
8. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood*. 1997;89:2079-2088.
9. Abramovich C, Pineault N, Ohta H, Humphries RK. Hox Genes: From leukemia to hematopoietic stem cell expansion. *Ann N Y Acad Sci*. 2005;1044:109-116.

10. Fathi AT, Abdel-Wahab O. Mutations in epigenetic modifiers in myeloid malignancies and the prospect of novel epigenetic-targeted therapy. *Adv Hematol*. 2012;2012:469592.
11. Wei Y, Gañán-Gómez I, Salazar-Dimicoli S, McCay SL, Garcia-Manero G. Histone methylation in myelodysplastic syndromes. *Epigenomics*. 2011;3:193-205.
12. Bacher U, Haferlach C, Schnittger S, Kohlmann A, Kern W, Haferlach T. Mutations of the TET2 and CBL genes: novel molecular markers in myeloid malignancies. *Ann Hematol*. 2010;89:643-652.
13. Bejar R, Levine R, Ebert BL. Unraveling the molecular pathophysiology of myelodysplastic syndromes. *J Clin Oncol*. 2011;29:504-515.
14. Raaijmakers MHGP, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464:852-857.

Owen A. O'Connor, Jennifer Amengual, Changchun Deng, Ahmed Sawas,
Bachir Alobeid, Daniela Hoehn, and Govind Bhagat

29 Lymphoma

The lymphoid malignancies comprise one of the most diverse and heterogeneous sets of diseases that exist under a single type of malignancy. In 2012, the American Cancer Society estimated there were about 70,000 cases of non-Hodgkin lymphoma (NHL), and about 10,000 cases of Hodgkin lymphoma in the United States, collectively accounting for about 4% to 5% of all cancers. The estimated number of U.S. cancer deaths attributed to lymphoma is about 3% for both males and females, ranking it about 8th among all causes of cancer death. Hodgkin lymphoma typically carries a better prognosis, accounting for approximately 1000 deaths per year. Whereas the lifetime risk of developing any cancer is about 1 in 2 for males and 1 in 3 for females, the lifetime risk of developing non-Hodgkin lymphoma is about 1 in 43 and 1 in 51 for males and females, respectively (for the years between 2006 and 2008). Despite the relatively lower incidence compared to other types of cancers, the trend in 5-year survival between 1975 and 2007 has been progressively improving. The trend in 5-year survival for all sites of cancer has improved between the years 1975 and 1977 (49%), 1987 and 1989 (56%), and 2001 and 2007 (67%), whereas for lymphoma in particular, for the same time periods, the 5-year survival reported by SEER is 47%, 51%, and 70%, demonstrating marked improvements in outcome over the past several decades. Many of these improvements stem directly from an improved classification system that has allowed for better risk stratification of patients, as well as the development of novel drugs targeting the unique pathogenetic features of the disease.

The classification of non-Hodgkin lymphoma has evolved since the first attempts at classifying these diseases were published. These classification schemes largely evolved from systems focused on morphology (large versus small cells, diffuse versus follicular patterns of lymph node growth), to ones that rely on immunohistochemical techniques to delineate lineage-specific markers, like those proposed by Luke and Collins and Kiel. The NCI Working Formulation attempted to integrate morphologic features and clinical presentation into the definition of subtypes, describing low-grade, intermediate, and high-grade lymphomas, where the grade refers to the aggressiveness of the clinical behavior, not the histologic grade of the tumor. The more recent classification systems as proposed by REAL and WHO represent fully integrated classification systems that take into account morphologic, immunologic, genetic, and clinical criteria to classify these complex diseases (Table 29-1). Increasingly, the integration of cytogenetic features has begun to subclassify discrete entities of these diseases, as has been demonstrated for the ALK^+ and ALK^- variants of anaplastic large T-cell lymphoma. In this case, the presence of the ALK translocation (t[2;5]) confers a highly favorable prognosis, which has a standard of care treatment recommendation (that is, CHOP-based chemotherapy); in contrast, the ALK^- ALCL, which is thought of more like peripheral T-cell lymphoma (PTCL), carries a very poor prognosis.

The lymphoid malignancies include a broad spectrum of diseases, including non-Hodgkin lymphoma, Hodgkin lymphoma, plasma cell malignancies such as multiple myeloma, and two forms of leukemia, namely, chronic lymphocytic leukemia (CLL) and acute lymphoblastic B- and T-cell leukemia/lymphoma (ALL) (though virtually any lymphoid malignancy can have a leukemic form of the disease) (Figure 29-1). These entities range from some of the fastest growing diseases known to science (such as Burkitt's lymphoma and blastic NK-cell lymphoma), to some of the slowest (CLL, marginal zone lymphoma). All lymphoid malignancies are derived from B-, T-, or NK lymphocytes originating in the primary lymphoid organs, such as the thymus and bone marrow (Figure 29-2). These lymphocytes are derived from pluripotent hematopoietic stem cells resident in the adult and fetal bone marrow as well as the fetal liver. Lymphoid malignancies derived from these less differentiated lymphocytes, before their differentiation in the thymus (for T-cell) or germinal center (for B cells), are considered undifferentiated lymphoid malignancies and typically give rise to diseases such as T- or B-cell acute lymphoblastic leukemia/lymphoma. Once the lymphocytes leave the primary lymphoid organs, they migrate to secondary lymphoid organs including the lymph nodes, tonsils, Peyer patches, spleen, and skin, where they undergo a multistep

Table 29-1 World Health Organization (WHO) Classification of Lymphoid Neoplasms (Partial List—Approximately 68 Types)

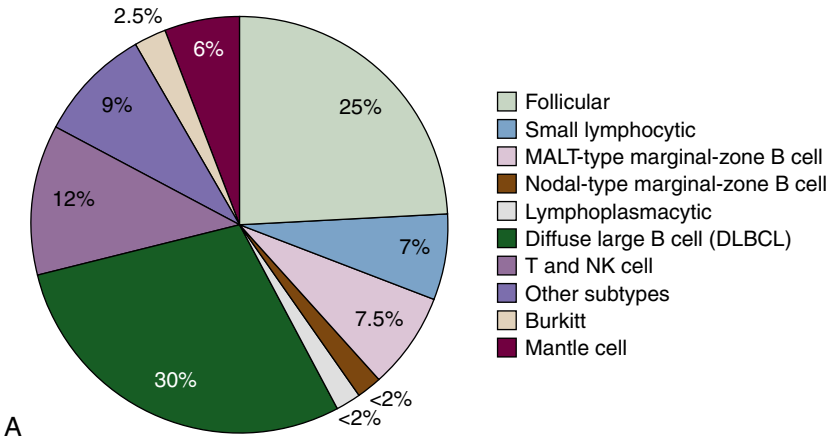
I. Precursor Cell Lymphoma
Lymphoblastic lymphoma, T-cell
Lymphoblastic lymphoma, B-cell
II. Peripheral B-Cell Lymphoma
SLL/CLL type
B-prolymphocytic leukemia
Lymphoplasmacytic lymphoma
Mantle cell lymphoma
Follicular lymphoma
Marginal zone lymphoma, MALT
Marginal zone lymphoma, nodal
Marginal zone lymphoma, splenic
Hairy cell leukemia
Diffuse large-cell lymphoma
Burkitt's lymphoma
III. Peripheral T and NK Lymphoma
T-prolymphocytic leukemia
Granular lymphocytic leukemia
NK cell leukemia
Mycosis fungoides/Sézary
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic lymphoma
NK/T cell, nasal
Enteropathy-associated lymphoma
Hepatosplenic γδ lymphoma
Subcutaneous panniculitis-like
Anaplastic large cell lymphoma, systemic
Anaplastic large cell lymphoma, cutaneous
Adult T-cell lymphoma/leukemia
IV. PTLD
Monomorphic
Polymorphic

process leading to their differentiation. Further differentiation of specific lymphocyte subsets then becomes more lineage dependent.

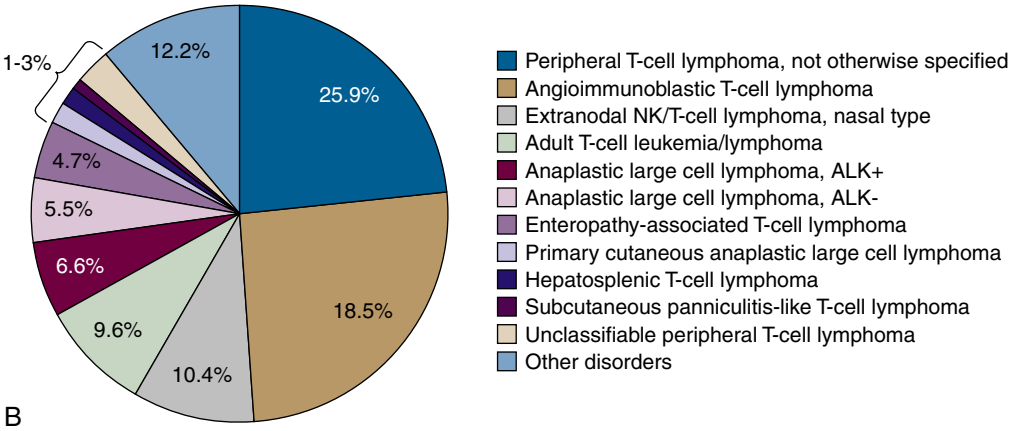
B- and T-Lymphocyte Development

The ontogeny of B cells is complex and proceeds through a series of developmental and genetic steps, leading to the creation of unique B cells carrying unique B-cell receptors, capable of identifying unique antigen (Figure 29-3). These developmental and genetic steps are defined by changes in the genome at the antibody loci. B lymphocytes play a fundamental role in the humoral branch of the adaptive immune system and can be distinguished from other types of lymphocytes by the panoply of cell surface markers (CD19, CD20, CD22, CD23) and the presence of the B-cell receptor (BCR), which itself is a surface immunoglobulin. Undifferentiated and immature B cells are produced in the intramedullary compartment of the bone. The antibodies produced by individual B cells are composed of two identical light chains (κ or λ), composed of two segments called the *V and J segments*, and one heavy chain (M, D, A, G) composed of three segments called the *V, D, and J segments*, where V denotes the variable region that comprises the terminal Fab portion of the antibody.[1] At the genetic level, early lymphocytes in the bone marrow undergo a process referred to as *VDJ recombination* (see Figure 29-2), which is the mechanism by which B cells produce immunoglobulin diversity. Once B lymphocytes undergo VDJ recombination, they exit the bone marrow, where they then hone to secondary lymphoid organs such as the lymph node. There they undergo highly controlled mutagenesis, in a process referred to as *somatic hypermutation* (SMH). SMH plays a crucial role in the affinity maturation of the humoral response, by mediating the introduction of discrete random mutations in the loci of the genes encoding the variable (V) regions of the immunoglobulin.[2] Once engaged by antigen, the subsequent proliferation of the B cell undergoes an incredibly high rate of somatic mutation that is at least 100,000- to 1 millionfold higher than the normal rate of mutation across the genome. This highly regulated mutagenesis, mediated initially by activation-induced cytidine deaminase (AID), introduces single base substitutions predominantly at hotspots in the DNA called *hypervariable regions*.[2] Paradoxically, it is this directed hypermutation of the BCR variable regions that allows for the selection of B cells with an enhanced capability to identify and bind specific foreign antigens, which, when dysregulated or uncontrolled, creates an opportunity for aberrant somatic hypermutation, leading to lymphomagenesis. This potential for any error, or error-prone mutagenesis of B lymphocytes, can create the background for malignant transformation.

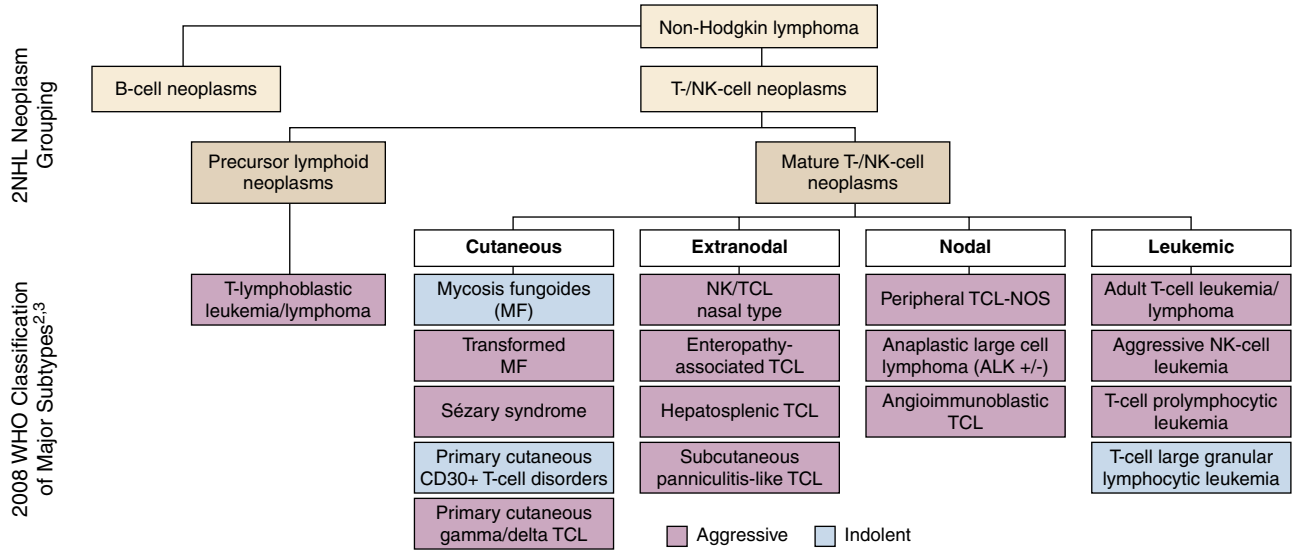
In contrast, T lymphocytes play a central role in cell-mediated immunity and can be distinguished from other lymphocytes by the expression of their own unique set of surface proteins (e.g., CD3, CD4, CD8) and the T-cell receptor (TCR). T lymphocytes typically mature in the thymus, in contrast to the germinal center for B lymphocytes. In the thymus, T lymphocytes expand through multiple series of cell division to produce a large population of more immature cells. Initially, these cells do not express CD4 or CD8 and are referred to as double-negative thymocytes.[3] As they differentiate into a specific subset of T lymphocytes, they eventually become positive for both CD4 and CD8 (that is, double positive). In their final step of thymic maturation they emerge as single positive T lymphocytes, including CD4-positive/CD8-negative T-helper cells or CD4-negative/CD8-positive cytotoxic T cells. The TCR recognizes antigen bound to major histocompatibility complex (MHC). Engagement of the TCR by antigen incites a number of downstream events important to the



A



B



C

Figure 29-1 **(A)** Relative incidence of select B- and T-cell lymphomas. **(B)** Relative incidence of select T-cell lymphomas. A survey of 1314 peripheral T-cell and natural killer/T-cell leukemia cases from 22 sites around the world evaluated the histopathology of previously untreated patients consecutively diagnosed between 1990 and 2002. PTCL-NOS was the most common subtype at 25.9%. Anaplastic large cell lymphoma (ALCL) ALK+/–, angioimmunoblastic lymphoma, adult T-cell leukemia/lymphoma, and natural killer/T-cell lymphoma were also common subtypes. **(C)** Classification of the mature T-cell lymphoma (WHO). PTCL refers to a heterogeneous group of aggressive T- and NK-cell lymphomas, characterized by the involvement of mature (thymic) T or NK cells. The term *peripheral* in PTCL does not refer to anatomic sites. T- and NK-cell lymphomas may be characterized as cutaneous, nodal, extranodal, or leukemic disseminated. *Data from Armitage JO et al.* Ann Oncol. *2004;15:1447-1449; Rodriguez J et al.* Crit Rev Oncol Hematol. *2008; Swerdlow SH et al.,* WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*; 2008. Data in* ***B*** *from Armitage J et al.* J Clin Oncol. *2008;26:4124-4130.*

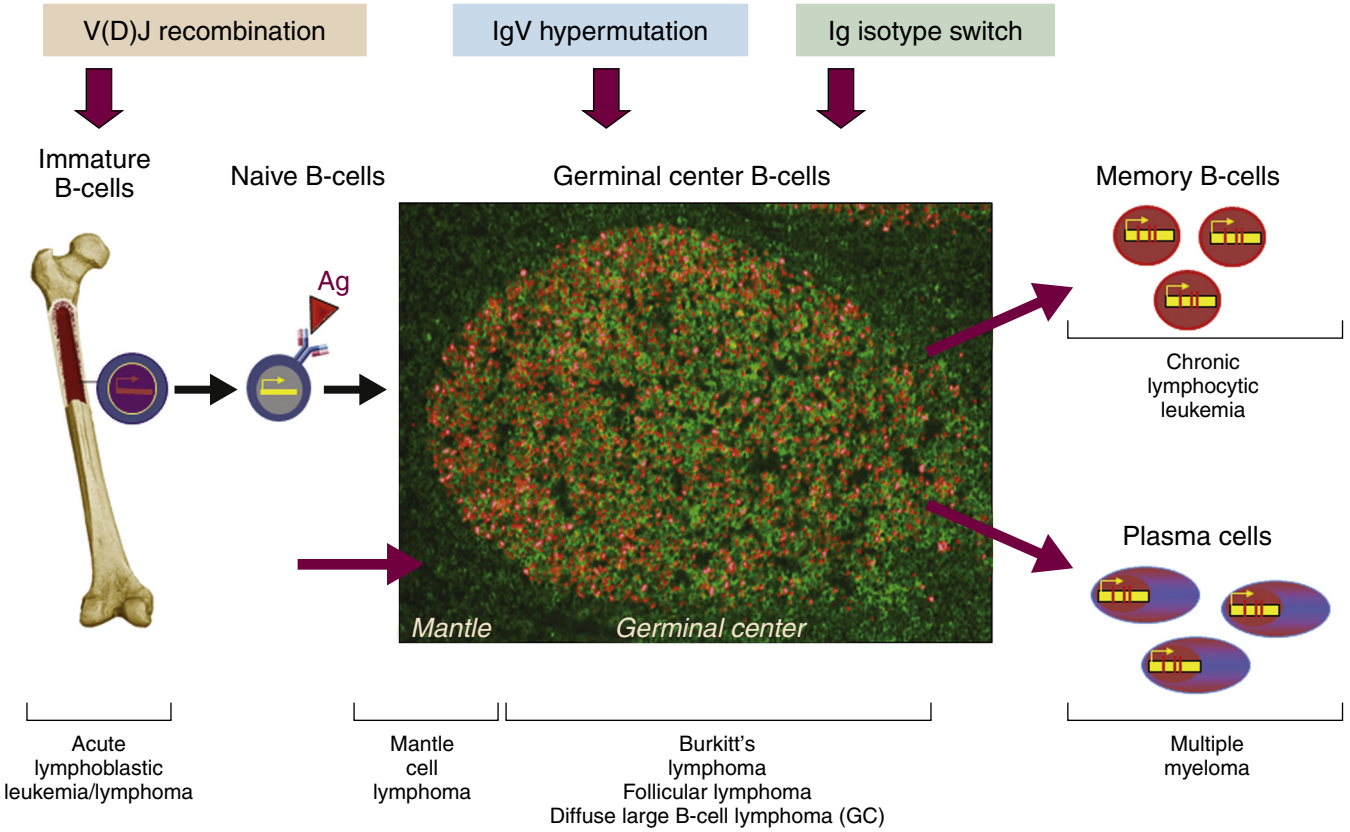


Figure 29-2 Schematic representation of the ontogeny of B-lymphocytes and select lymphoma relative to their sites of origin.

lymphocytes role in mediating cellular immunity. The generation of the TCR is quite similar to that discussed earlier for the BCR. Composed of four chains and existing as a dimeric structure (alpha pairing with the beta chain or the gamma chain pairing with the delta chain), unique TCR is produced through VJ (the alpha and gamma chains) or VDJ (the beta and delta chains) recombination. Like the heavy and light chains comprising the BCR, it is the unique combination of these specific regions that accounts for the diversity of the TCR.[3]

Although complex, the ontogeny of B and T lymphocytes provides the basis for thinking about the diversity of different forms of non-Hodgkin and Hodgkin lymphoma, and in particular the cell of origin (see Figure 29-3). In the case of the lymphoid malignancies, identifying the cell of origin goes well beyond the identification of the lymphocyte and is ascribed to the discrete step in development at which the clonal expansion is thought to occur. Increasingly, the understanding of immunohistochemical staining and cytogenetics has allowed for a description of the natural ontogeny of lymphocytes and, by extension, the more accurate identification of the cell of origin, leading to a more biologically relevant classification of different subtypes of lymphomas (Table 29-2).

Pathogenesis of Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) comprises a group of aggressive lymphomas with very heterogeneous clinicopathologic and molecular genetic features. The current 2008 World Health Organization (WHO) classification[4] defines DLBCL as a diffuse growth of neoplastic large B-lymphoid cells with a nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte. DLBCL is the most common subtype of lymphoma and accounts for 30% to 40% of adult NHL.[5] The 2008 WHO classification recognizes a large number of DLBCL subgroups primarily based on their distinct morphologic, biologic, immunophenotypic, or clinical features. Despite revisions to the classification, a significant proportion of large-cell lymphomas remain biologically heterogeneous, not fitting into any specific DLBCL subgroup. These are typically defined as DLBCL, not otherwise specified (DLBCL-NOS). DLBCL-NOS is a diagnosis of exclusion, not corresponding to one of the specific subtypes. It is still the most common type of lymphoma, accounting for 25% to 30% of NHLs.[5]

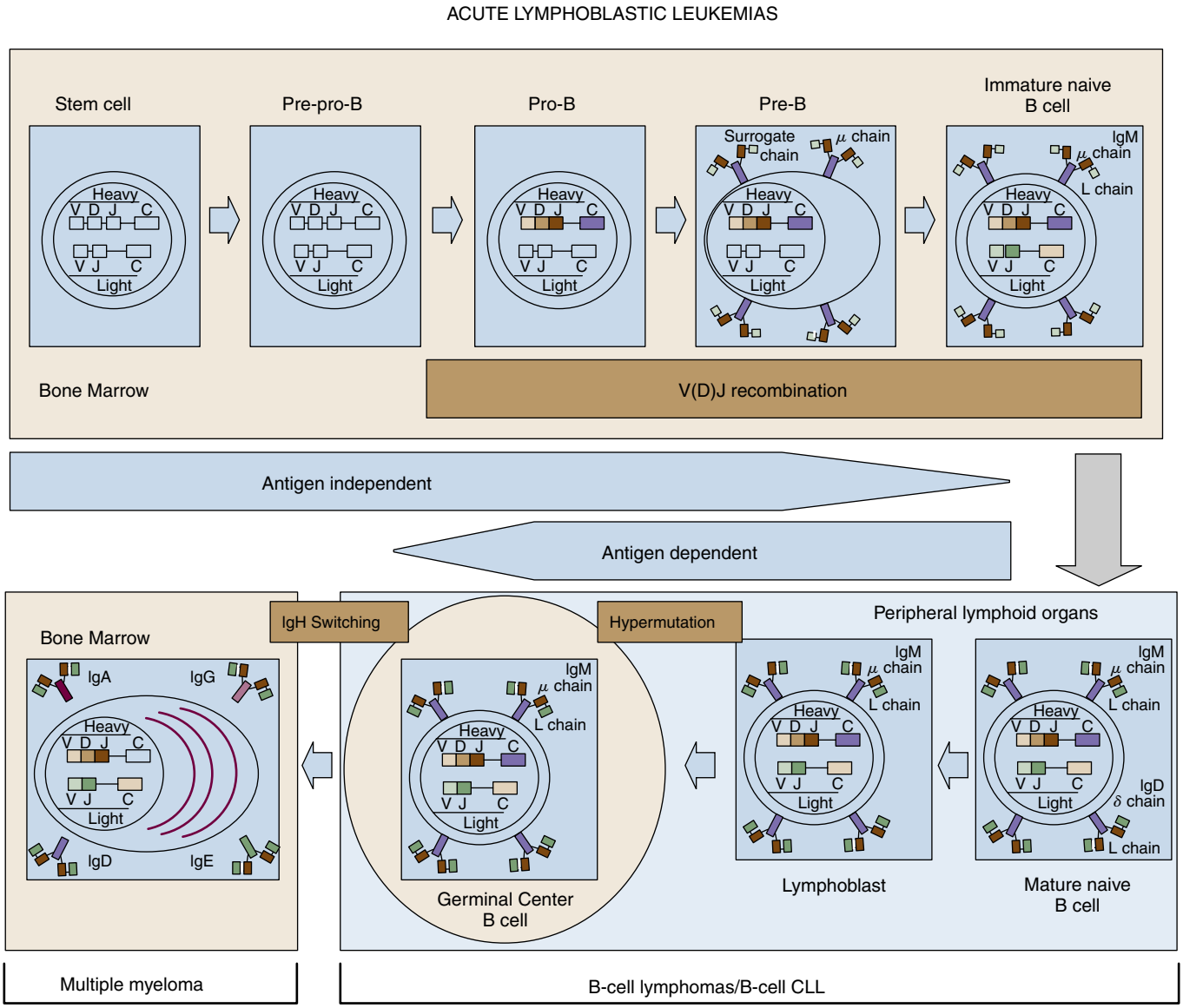


Figure 29-3 Stages of B-cell development and correlation with different lymphoid diseases. *CLL*, Chronic lymphocytic leukemia.

The focus in recent years has shifted toward the identification of molecular alterations and specific pathogenetic pathways leading to transformation in DLBCL. Gene expression profiling (GEP) of DLBCLs has identified at least two major molecular subtypes, which correlate with prognosis and may have relevance for treatment based on the dominant signaling pathways. DLBCL could be classified by GEP as resembling either germinal center B cells (GCBs) or activated B cells (ABCs), establishing a putative "cell of origin"[5] (Figure 29-4). Because GEP is technically difficult and therefore cannot be performed in every case for diagnostic purposes, various algorithms based on immunohistochemical profiles have been proposed as surrogates of the GEP.[5,6] Although the correspondence is not precise, similar prognostic correlations can be drawn with immunohistochemically defined subgroups. These immunohistochemical algorithms have facilitated risk stratification of DLBCL patients and DLBCL research using archival materials.[5]

The ABC and GCB DLBCL subtypes, originally formulated based on a cell-of-origin model, have more recently been shown to be characterized by the different pathways of cellular transformation and oncogenesis. Although substantial progress has been made toward molecular subclassification of DLBCLs, the translation to effective treatment strategies is only now beginning to be explored.[5] Next-generation sequencing (NGS) platforms have evolved to provide an accurate and comprehensive means for the detection of molecular mutations and will likely contribute to a more sophisticated understanding of DLBCL biology and, it can be hoped, more biologically relevant treatment.[7]

Table 29-2 Characteristic Immunophenotypic and Genetic Features of Selected Lymphoma Subtypes

Lymphoma Subtypes	Immunophenotypes	Frequent Translocations/ Aberrations	Genes Involved	SHM	Ongoing SHM	Putative Cell of Origin
GC B-cell–like DLBCL	CD20, CD22, CD79a, BCL-2 CD10	t(3q)(27) → t(14;18) → t(8;14) →	BCL6 (35%) BCL2 (15-30%) MYC (15%)	√ √	√ –	GC B cell GC B-cell subset or extra-GC mutated B cell
Activated B-cell–like DLBCL	MUM1	t(3q)(27) gain18q del6q gain3p del9p21 gain/amp19q	BCL6 (25%) BCL2, NFATC1 (40%) PRDM1 (BLIMP1) (25-30%) FoxP1 (25%) CDKN2A (30%) SPIB (25%)			
Follicular lymphoma (FL)	CD20, CD22, CD79a, CD10, BCL-2, BCL-6	t(14;18) →	BCL2 (90%)			GC B cell
MALT lymphoma	CD20, CD79a; negative for CD10 +/- CD 5 and CD23	t(11;18) → t(14;18) t(3;14) t(1;14) → +3/3q and/or +18 del6q	API2-MALT1 (30%) IGH-MALT (5%) IGH-FOXP1 (10%) BCL10 (1%) - (40%) TNFAIP3 (A20) (30%)	√	√	GC B cell or post-GC B cell
Mantle-cell lymphoma	CD20, CD79a, CD5; negative for CD10, CD23	t(11;14) →	Cyclin D1 (95%)	–	–	Pre-GC B cell
Burkitt's lymphoma	CD20, CD10, BCL-6	t(8;14) →	MYC (100%)	√	√	Pre-GC B cell
Classical Hodgkin lymphoma	CD30, CD15 (CD79a) PAX5; MUM1	Gain/amp2p t(16p)(13)	REL (54%) CIITA (15%)	√	–	GC or post-GC B cell
Nodular lymphocyte predominant Hodgkin lymphoma	CD20, CD79a, BCL-6, CD45, CD75, OCT2	t(3q)(27)	BCL6 (50%)	√	√	GC B cell

DLBCL, Diffuse large B-cell lymphoma; *GC*, germinal center; *MALT*, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissues; *SHM*, somatic hypermutation.

Although increased understanding of DLBCL based on cell of origin is emerging, it is clear that the dysregulated pathways across this entity are often overlapping, providing redundancy to promote growth and prevent cell death signals. These signaling pathways are often necessary for the development and differentiation of normal memory B cells and plasma cells and are coopted during malignant transformation.

For example, in ABC-derived DLBCL, most of the deranged signaling pathways converge to aberrantly activate the NFκB pathway, leading to evasion of cell death and resistance to chemotherapy (see Figure 29-4, *B*). Activation of this molecular pathway translates into poor outcomes for patients with ABC-DLBCL compared to GC-DLBCL, with 5-year overall survival of 40% versus 60%, respectively. Constitutive activation of NFκB is complex, involving both "classical" and "alternative" pathways, and can be regulated by many different mechanisms. Derangements leading to activation of NFκB can be attributed to a number of different molecular lesions, including tonic signaling of the BCR. BCR signaling is essential to B-cell development, antigen selection, and humoral immunity. Aberrations in BCR signaling, including tonic signaling that occurs in the absence of antigenic stimulation, can lead to uncontrolled B-cell growth and survival.

BCR signaling, whether it be in a normal or malignant B-cells, leads to the formation of the CBM complex consisting of CARD11, *B*CL10, and *M*ALT1. This complex leads to activation of NFκB through direct activation of NFκB and inhibition of negative NFκB regulators. Gain-of-function somatic mutations of the coiled-coil domain of CARD11 are found in nearly 10% of ABC-DLCBL and 16% of primary CNS lymphoma (an ABC variant). This mutant CARD11 leads to initiation of the NFκB cascade via IKKβ signaling in cooperation with casein kinase 1α (CK1α) and MALT1. MALT1 can also augment NFκB signaling through cleavage of two negative NFκB regulators, namely, A20 and CYLD. *TNFAIP3*, the gene encoding A20, can also be inactivated through mutations, deletions, or epigenetic silencing in ABC-DLCBL. Mutations of other negative regulators of NFκB, such as *IKBKA* encoding the inhibitor IkBα, lead to uninhibited translocation of NFκB to the nucleus and increased transcription of NFκB-dependent genes. Interestingly, linkage of this intrinsic ABC biology to B-cell receptor signaling may offer an interesting opportunity to affect the adverse prognostic features of this DLBCL phenotype.

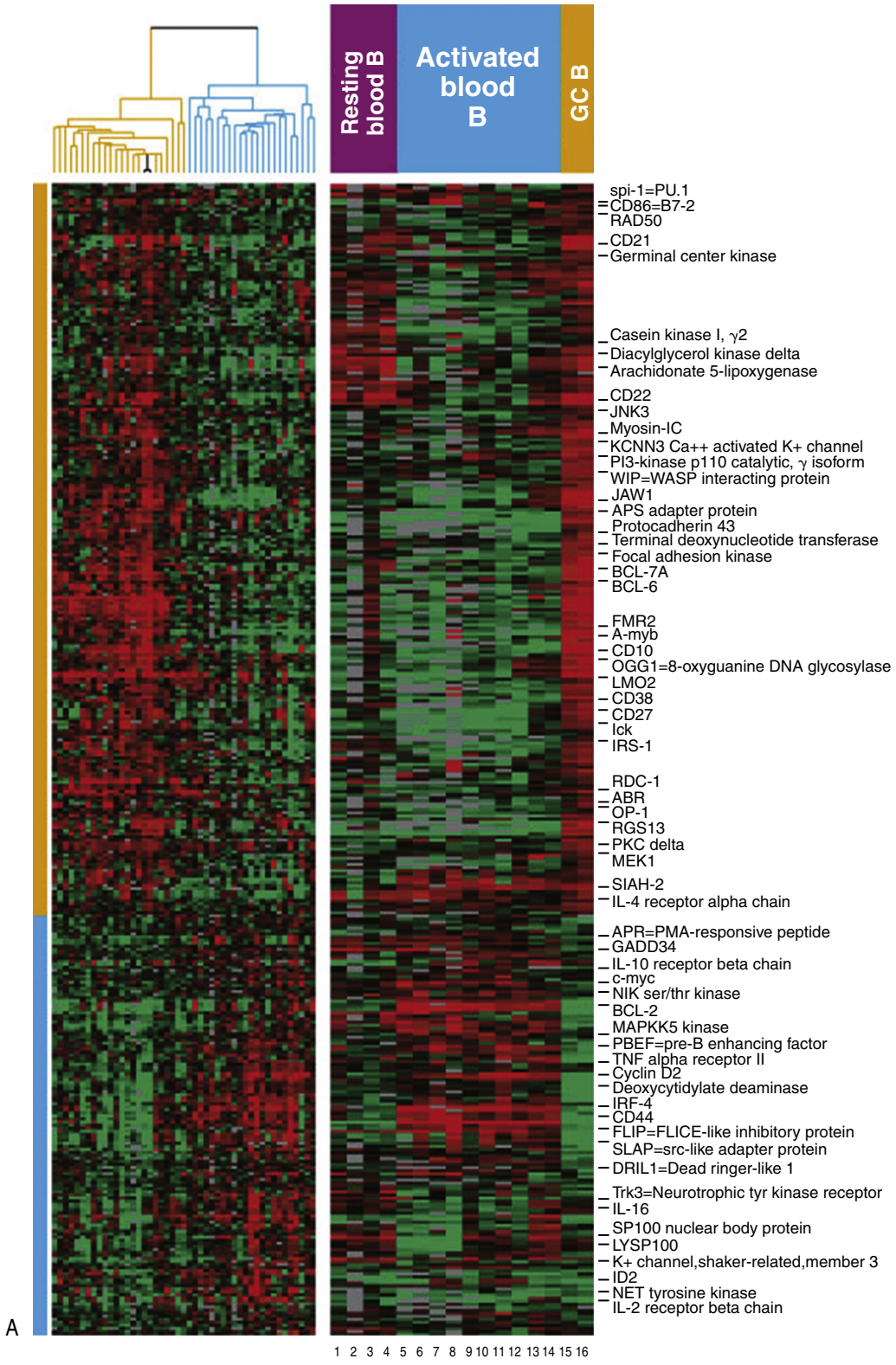
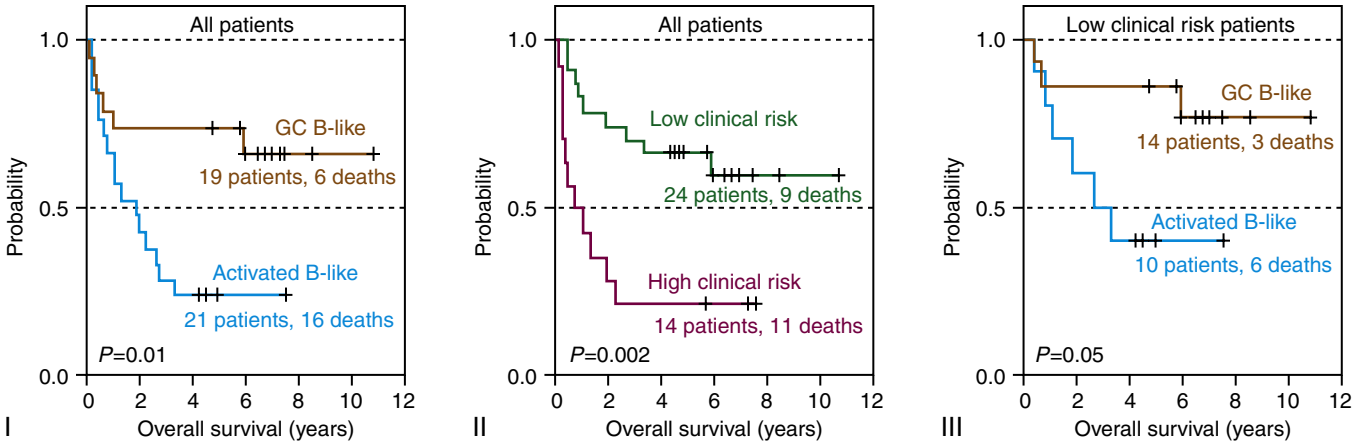


Figure 29-4 (A) Classification of diffuse large B-cell lymphoma based on cell of origin. The left panel represents hierarchical clustering of the genes selectively expressed in GC-derived DLBCL *(yellow)* and ABC-derived DLBCL *(blue)*. The right panel compares normal lymphocytes from blood and lymph nodes in various states of B-cell activation.

Continued



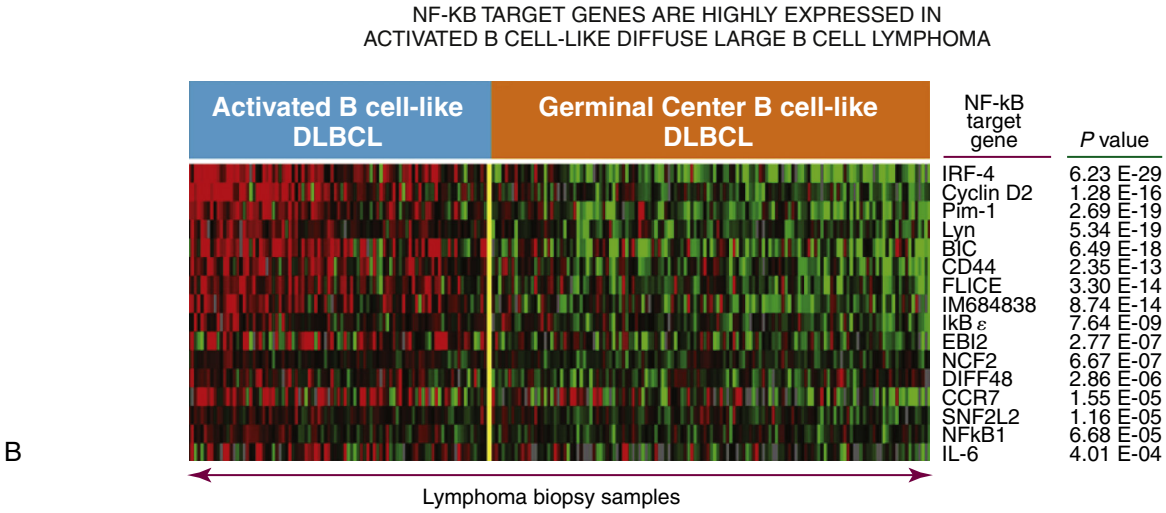


Figure 29-4, cont'd **(B)** Gene expression profile for the GC and ABC subtypes of DLBCL reveal that the ABC phenotype is enriched for NFκB-regulated genes, while the GC phenotype is enriched for Bcl-6-regulated genes. Gene expression profiling has revealed three subtypes of DLBCL, two of which include the germinal center subtype (GC) and the activated B-cell subtype (ABC). These molecular phenotypes override the prognostic impact of the International Prognostic Index (IPI). *Courtesy Wyndham Wilson. Data in* ***A*** *from Alizadeh et al.* Nature *2000;403:503-511.*

In addition to the BCR-CBM effects on NFκB regulation, the BCR can activate NFκB through a number of alternative mechanisms. On antigenic stimulation or tonic dysregulation, the BCR heavy chains are coupled to cell surface markers CD79a and CD79b. This leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) by LYN, FYN, and BLK, which then recruit SYK, engaging the NFκB, PI3K, NFAT MAP kinase and RAS signaling pathways. Currently, an inhibitor of SYK is under investigation as a therapeutic target in those DLBCLs known to be addicted to BCR signaling. Substitutions and deletions of the CD79a and CD79b ITAMs are present in more than 20% of ABC-DLBCL and lead to a gain of function of the BCR signaling pathway. These mutants evade negative feedback by LYN and also allow for increased cell surface localization of the BCR.

Tonic BCR signaling also recruits Bruton's tyrosine kinase (BTK) via the PI3K pathway. This tends to occur in ABC-DLBCL that do not possess CARD11 mutations. BTK is required for the survival of wild-type CARD11 ABC-DLBCL and knockdown of its components or effectors SYK, BLNK, PLCγ2, PI3Kδ, PKCβ abrogates NFκB signaling. BTK is activated by LYN or SYK, and on activation initiates signaling of the classical NFκB pathway. In addition, BTK forms a complex with BLNK, which, through a series of events that activates CARD11, contributes further to activation of the NFκB pathway. BTK inhibitors have demonstrated promising activity in patients with ABC-DLBCL and other lymphomas with aberrant BCR signaling, such as CLL.

All known ABC-DLBCL cell lines and 30% of tissue samples from patients with ABC DLBCL harbor MYD88

gain-of-function mutations in the toll-interleukin receptor (TIR) domain. MYD88 is an adaptor protein that interacts with IRAK4 and IRAK1 to activate both the NFκB and interferon pathways through TRAF6. Mutant MYD88 promotes secretion of the cytokines IL6 and IL10 activating the JAK/STAT pathway. This potentiates NFκB through a positive feedback loop.

Many of the mutations just discussed are expressed in tandem and are restricted to the ABC-DLBCL variant over GCB-derived DLBCL. As testament to the redundancy in these signaling pathways, knockdown of parallel pathways in cell lines or mouse models has demonstrated synergistic cytotoxicity in ABC-DLBCL, compared to the knockdown of singular elements in a pathway. The clarification of this underlying biology has led to the identification of several new drugs potentially affecting these specific pathways, including inhibitors of Syk, BTK, and CD79a and b, and proteasome inhibitors that inhibit degradation of IkB, leading to inhibition of NFκB.

Although there is overlap in the pathogenesis of GC- and ABC-derived DLBCL, there are unique features of both that may create opportunities to affect the discrete natural history of these subtypes rationally. For example, transcription factors, oncogenes, and epigenetic alterations are often responsible for driving proliferation and survival in GC-DLBCL. *BCL6*, an oncogene, is a transcriptional repressor considered the hallmark of GC-DLBCL, and acts as a master regulator of the germinal center reaction. Although physiologically active in normal germinal center B cells promoting somatic hypermutation (SMH) and class switch recombination, this pathway is co-opted in malignant lymphomas to enforce the "damage phenotype" of GC-DLBCL. Dysregulation through point mutations and translocations of the 3q27 locus lead to constitutive activation of this transcription factor in over 50% of DLBCL lymphomas. Specifically, mutations in the BCL6 autoregulatory domains upstream from the promoter regions via somatic hypermutation and AID result in lost feedback loop inhibition from Bcl6, IRF4, and STAT5. This phenomenon leads to the GC-DLBCL subtype. In addition, the Bcl6 BTB domain recruits the co-repressors NCOR, BCOR, and SMRT, along with HDACs to repress genes involved in the DNA damage response such as ATR, p53, CHK1, p21, and p27. This repression leads to unchecked cell cycle progression in the face of inhibited apoptosis and permissive DNA damage. Furthermore, the Bcl6 RD2 domain recruits MTA3 to repress *PRDM1*, which encodes Blimp-1, the master regulator of plasma cell development, trapping centroblasts within the germinal center. Interestingly, Bcl6 inhibits both *MYC* and *BCL2*, but in double-hit lymphomas, where these two oncogenes are overexpressed by translocations, they overcome repression by Bcl6, leading to even more aggressive variants of DLBCL.

Another target gene of Bcl6 is IRF4, a transcription factor that facilitates exit from the germinal center, leading to plasmacytic differentiation. Bcl6 silencing of IRF4 further inhibits transcription of Blimp-1, which would retain cells in the germinal center. IRF4 may also be inhibited by translocations in GC-DLBCL (predominantly pediatric cases). In these cases, IRF4 may serve as an oncogene. Nonstructural aberrations not interfering with IRF4 function have been found to be required for survival of ABC-DLBCL. The effects of IRF4 in ABC and GC-DLBCL are clearly different, and this likely contributes to their classification by cell of origin. One mechanism proposed is that IRF4 works as a positive feedback loop involving CARD11.

Although Bcl6 translocations occur in GC-DLBCL (10%), translocations of the 3q27 locus involving the BCL6 gene that lead to arrest of cells in the plasmablastic stage of development can be seen in 30% of ABC-DLBCL. These translocations have promiscuous partners and ultimately lead to silencing of *PRDM1*. The tumor suppressor functions of *PRDM1* may be silenced by truncation, deletion, mutation, or epigenetic modifications as well.

Transcriptional control has also been influenced by mutations in epigenetic regulators. *CREBBP* and *EP300* encode histone acetyl-transferases (HATs) that have classically been known to modify chromatin condensation and, by extension, transcription. It is now known that these enzymes also influence the activity of key modulators of DLBCL such as p53, NFκB, and Bcl6. Acetylation can act either as an activating posttranslational modification (p53), or as an inhibitory posttranslational modification (Bcl6). Nonoverlapping mutations of *CREBBP* (40%) and *EP300* (10%) are found in GC-DLBCL. The inactivating mutations in these two genes lead to the activation of Bcl6 and inhibition of p53 by impaired acetylation. Reversal of this effect may be accomplished by therapeutic alteration of these posttranslational modifications pharmacologically with pan-Class1-2 histone deacetylase (HDAC) inhibitors.

GC-DLBCL has also been found to have mutations in the EZH2 component of the polycomb repression complex-2 (PRC2). This complex has histone methyltransferase activity and primarily trimethylates histone H3 on lysine 27 (i.e., H3K27me3), a mark of transcriptionally silent chromatin. In order to produce the pathologic effect, mutations are required to be heterozygous, allowing the wild-type allele to methylate H3K27, followed by the uncontrolled methylation mediated by the enzyme encoded by the mutant allele. This action leads to hypermethylated CpG islands, which in turn attracts methyl-CpG binding domain proteins (MBDP: MBD1, MBD2, MBD3, MeCP2 i Kaiso) to recruit HDACs, leading chromatin condensation and transcriptional silencing. Hypomethylating drugs have been shown in preclinical models to have therapeutic potential

and synergize with HDAC inhibitors to restore normal methylation and acetylation patterns on histones.

Although only recently described, the role of microRNAs in DLBCL lymphomagenesis has begun to emerge. MiRNAs can have both oncogenic and tumor suppressor properties. The miR-17-92 family, also known as oncomir-1, has been found to be amplified specifically in GC-DLBCL, which leads to increased expression of MYC and downregulation of BIM and p21. miR-17-92 also has an inverse relationship with p53, each inhibiting the other.

Molecular Pathogenesis of Follicular Lymphoma

Follicular lymphoma is a B-cell lymphoma derived from germinal center B cells with rearranged heavy- and light-chain genes. This derivation is depicted by the immunoarchitectural features of follicular lymphoma, particularly reflected in the expression of a germinal center immunohistochemical profile as well as a follicular growth pattern. Cytogenetically, about 90% of follicular lymphoma cases harbor the t(14;18) or variant B-cell CLL/lymphoma 2 *(BCL2)* rearrangements with immunoglobulin kappa or lambda on chromosome 2 and 22, respectively. Although centrocytes and centroblasts are regarded as the particular cell of origin, the lymphomatous counterpart demonstrates ongoing somatic hypermutations, which is of particular interest as it decreases the detection rate of follicular lymphoma by standard polymerase chain reaction (PCR) testing.

The most prominent cytogenetic abnormality in follicular lymphoma involves t(14;18) with rearrangement of *BCL2*. Normal BCL2 protein has an antiapoptotic effect by affecting mitochondrial permeability and release of cytochrome c. During the translocation of the IgH regulatory apparatus on chromosome 14 and the bcl-2 gene on chromosome 18, *BCL2* is juxtaposed to immunoglobulin heavy-chain gene (IgH) sequences, including enhancer sequences, which brings *BCL2* under the influence of the immunoglobulin promoter region. This leads to deregulated expression/overexpression of a functional protein, promoting increased cell survival and neoplastic transformation.

The rearrangement can occur in different breakpoint regions as noted:

- 65% to 70% occur in the major breakpoint region (MBR) located in an untranslated region of the last exon (exon 3).
- 20% occur at the 3′ end of the *BCL2* gene or 5′ to the minor cluster region (mcr).
- 10% occur in the mcr, which is about 30 kb outside of the *BCL2* gene.

Although additional breakpoints are being studied, efforts to associate these different breakpoints with different clinical characteristics or clinical outcome have been unrevealing.[8]

Testing for the *BCL2* rearrangement can be determined by conventional cytogenetic karyotyping, fluorescence in situ hybridization (FISH), or PCR. Although conventional cytogenetic karyotyping detects the majority of cases with *BCL2* rearrangement, independent of the partner gene, the limiting factor with this approach remains the availability of viable tissue. In comparison, FISH can be performed on paraffin-embedded tissue and detects the translocation in up to 90% of cases. Lower detection rates have been reported depending on the ethnic group studied—such as in Japanese patients, where the frequency of the t(14:18) has been shown less frequently compared to patients from Western countries.[9] Of interest, PCR testing has been proven to be less valuable, as most PCR assays contain primers targeting the MBR and mcr regions, which decreases the detection rate to 60%.[10] Although increased detection rates can be achieved by adding more primers to the 5′ mcr and 3′ MBR breakpoint regions, targeting incomplete DH-JH rearrangements or mutations of the primer-binding sites, false-negative PCR results are common and still occur with alternate breakpoints as mentioned earlier.[11] Therefore, up-to-date FISH analysis remains the preferred testing modality.[11]

The absence of *BCL2* translocation using the routinely available methods is described in a subset of follicular lymphomas, particularly primary cutaneous and pediatric follicular lymphoma. Further complicating the matter is the fact that multiple follicular lymphoma cases have been shown to increase their *BCL2* expression through alternate mechanisms, as shown in cases with an addition of 18q or trisomy 18.[12] Furthermore, the frequency of *BCL2* rearrangement is not only variable in different subsets of follicular lymphomas, but also differs as a function of the grade of the follicular lymphoma. More than 80% of low-grade follicular lymphoma cases possess the translocation, whereas higher grade follicular lymphoma cases harbor the translocation with a frequency of 70% and less than 15% in grades 3A and 3B, respectively.[13] However, a positive result for *BCL2* rearrangement demands additional studies, as the rearrangement is not specific and has been identified in other subtypes of lymphomas as well as in healthy individuals and in benign follicular hyperplasia.[14]

An additional random locus of genetic alteration in follicular lymphoma includes alterations of BCL6 at 3q27. Grade 3B follicular lymphomas frequently have rearrangements of this locus similar to diffuse large B-cell lymphoma.[13,15] The Bcl6 protein is a zinc-finger protein that acts as a repressor of IRF-4/MUM-1 transactivating ability, as well as influencing cell cycle control and proapoptotic

genes. The major breakpoint region of this translocation is within the 5′ first exon and a portion of the first intron of the Bcl-6 gene. In addition to translocation events, the coding region of the Bcl6 gene is also altered by somatic mutation.

Because t(14;18) can be identified in healthy individuals, the translocation is thought to be an early event in B-cell development, neither necessary nor sufficient for lymphomagenesis in B cells. The *BCL2* rearrangement is thought to enhance the life span of the neoplastic cell, which in return retains the capability to develop additional genetic defects. So far a myriad of additional abnormalities have been described, including gains of 1q, 2p, 7, 8q, 12q, 18q, and X and losses of 1p, 6q, 10q, 13q, and 17p.[16]

After detection of these additional abnormalities, prognostic factors have been identified, including gains of 1q, 12, and X, as well as losses of 1p, 17p, and 17q, all considered late events with a poor prognosis or associated with histologic transformation.[16] Transformation to diffuse large B-cell lymphoma is a relatively common event, occurring in 25% to 35% of follicular lymphoma cases.[16] To date, large-cell transformation has been linked to gains of 7, 12q, and X; losses of 4q, 13q, and 17p; *MYC* deregulation; and inactivation of *TP53* and *CDKN2A*. Although *MYC* deregulation has only been occasionally described in follicular lymphoma, it harbors a particularly poor diagnosis.[17] Newer diagnostic modalities such as comparative genomic hybridization (CGH) have firmly established that additional chromosomal abnormalities, such as gains in 12p and 18p in more than 10% of cases and deletions of 3q, 9p, and 11q in less than 10% of cases, are associated with particularly poor prognostic factors. Although these deletions involve the *CDKN2A* and *CDKN2B* loci as well as the LIM domain and have been partially associated with decreased survival, the overall significance of these findings has yet to be determined.[18]

Next-generation sequencing has been used to elucidate the mutational landscape of follicular lymphoma. With this modality it was discovered that 89% of cases harbored MLL2 mutations, 33% of cases showed CREBBP mutations, and 13% of cases depicted MEF2B mutations. E300 mutations were identified in 9% of cases, EZH2 mutations in 7% of cases, and the TBL1XR1/TP63 fusion gene was identified at a very low incidence. So far mainly the presence of EZH2 is of interest, as this particular mutation supports the hypothesis that the polycomb repressor complex-2 orchestrates the pathogenesis in a subset of germinal center–derived lymphomas, but in general the implications of these findings are still under investigation.[19]

Additional factors thought to be involved in the pathogenesis of follicular lymphoma include the nature of the microenvironment. In particular, the role of T cells and dendritic cells appears to be of substantial importance. GEP of the microenvironment has identified two distinct signatures. These signatures have been categorized as immune response 1 (IR1) and immune response 2 (IR2). Whereas immune response 1 (IR1) shows increased expression of T-cell genes and the macrophage genes *TNFSF13B* and *ACTN1*, immune response 2 (IR2) shows increased expression of follicular dendritic cell genes and different macrophage genes. Immune response 1 has been linked to a more favorable prognosis, but immune response 2 has been linked to an unfavorable prognosis.[16] To date, these genetic changes have not yet led to the tailoring of specific therapies for specific genetic subsets of the disease.

Molecular Pathogenesis of Marginal Zone Lymphoma and Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Marginal zone lymphoma (MZL) arises from memory B cells that normally reside in the marginal zone of secondary lymphoid follicles. MZL is the third most common type of non-Hodgkin lymphoma, representing about 10% of all cases of NHL. At least three distinct entities of MZL have been included in the WHO classification, including extranodal MZL of mucosa-associated lymphatic tissue (MALT) lymphoma, which represents more than two thirds of all MZL cases; splenic MZL with circulating villous lymphocytes; and nodal MZL. Clinically, MZL typically presents as early-stage disease in most patients and exhibits an indolent course in most patients. However, MZL can become disseminated or transform into aggressive large-cell lymphoma. Over the past two decades we have witnessed unprecedented progress in our understanding of the biology of MZL at the molecular level, and some of that knowledge is being translated into the development of rational treatment paradigms.

Because MALT lymphoma develops in tissues that are not necessarily abundantly enriched with lymphocytes, the first step in the pathogenesis is the abnormal aggregation of lymphoid tissues in extranodal sites and organs. The etiologic factor in this process can be infectious and/or inflammatory. Gastric MALT lymphoma represents more than 50% of all MALT lymphoma and has been shown to be intricately associated with colonization of the stomach by the bacterium *Helicobacter pylori*.[20] Although aggregation of lymphocytes stimulated by *H. pylori* is a prerequisite for MALT lymphoma, *H. pylori* infection alone is not sufficient to cause the malignant transformation of lymphocytes into lymphoma cells. *H. pylori* is found in more than 90% of patients with MALT lymphoma, yet most patients with *H. pylori* infection do not develop lymphoma. *H. pylori* also produces inflammation and reactive oxygen species (ROS)

that further increase genomic instability, which is essential for lymphomagenesis. In support of the theory of genomic instability in gastric MALT lymphoma, a balanced translocation t(11;18)(q21;q21) was reported in up to 50% of all cases of the disease.[21] The translocation results in the production of fusion protein API/BIRC3-MALT1, which interacts with CARD11/CARMA1 to activate the critical pro-survival transcription factor NFκB.[22] The translocation t(11;18) has been found to be mutually exclusive of other cytogenetic abnormalities, further suggesting its role as an important, if not the singular, driver for the development of MALT lymphoma.[23] Importantly, the emergence of t(11;18) is associated with unfavorable clinical outcomes and resistance to antibiotics and chemotherapy in the treatment of gastric MALT lymphoma.[24]

In addition to its occurrence in the gastrointestinal tract, MALT lymphoma has been reported in the lung, ocular adnexa, salivary gland, and less commonly the skin and thyroid. MALT lymphoma in these sites often involves a different etiology. For example, *Chlamydophila psittaci* or *Borrelia burgdorferi* is thought to be responsible for MALT lymphoma of the ocular adnexa and skin, respectively, whereas inflammation caused by autoimmune disease is responsible for MALT lymphoma in the thyroid and salivary gland. Cytogenetic abnormalities are well described for MALT lymphoma in these sites and include t(14;18) and t(1;14), which involve MALT1 and BCL10, respectively. Other genetic abnormalities in MALT lymphoma include t(3;14) involving IGH-FOP1, +3/3q, +18/18q, and −6q23, many of which target TNFAIP3 (A20), a negative regulator of NFkB, which is also mutated and silenced by methylation in subsets of MZL.[25] The pathogenetic role of many of these genes and loci are poorly understood at present.

Splenic MZL is a less common entity of MZL and has been shown to be associated with hepatitis C infection (HCV) in some cases.[26] In these cases, HCV glycoprotein E2 interacts with CD81 on B cells and causes activation of the BCR and its downstream signals.[27,28] Similar to other etiologic factors in MZL, HCV is required for the initiation and maintenance of the malignant phenotype of MZL, but not sufficient to cause malignant transformation of memory B cells in the marginal zone. This is evident from the fact that most patients with HCV do not develop splenic MZL. Furthermore, the majority of patients with splenic MZL are not associated with HCV. Clearly, genetic and epigenetic alterations are required for the development of splenic MZL. Although t(11;18) is not associated with splenic MZL, cytogenetic abnormalities are very common in splenic MZL, including partial trisomy of 3q, gain of 12q, and deletion or translocation of 7q32.[21,29] Furthermore, recent studies have demonstrated that deregulation of the NFκB pathway is frequently observed in splenic MZL. For example, mutations and copy number abnormalities of NFκB genes have been shown to occur in 36 (36%) of 101 splenic MZLs in one series; in another series, inactivating mutations were found in the NFκB negative regulator A20 (6/46 = 13%), and activating mutations were found in MYD88 (6/46 = 13%) and CARD11 (3/34 = 8.8%). Interestingly, these mutations are largely mutually exclusive of each other, suggesting that they may play vital roles in the pathogenesis of splenic MZL. Mutations in NOTCH2, a gene required for marginal-zone B-cell development, is a recurrent genetic lesion in splenic MZL, accounting for approximately 20% to 25% of cases.[30] Importantly, NOTCH2 mutations are restricted to splenic MZL but not other indolent B-cell lymphoproliferative disorders such as MALT lymphoma.[31] These mutations are predicted to cause impaired degradation of the NOTCH2 protein or may result in a gain of function of the truncated NOTCH2 in splenic MZL. The 7q deletion mentioned earlier was investigated recently using a combination of high-resolution array comparative genomic hybridization and integrated transcriptomic analysis. No single gene from these analyses could be identified as a potential driving event for splenic MZL with del7q.[32] Rather, a number of genes and microRNA species were found to be downregulated, some attributed to promoter hypermethylation. Another study of the 7q deletion arrived at a similar conclusion and was able to find nucleotide changes in IRF5 in two of the patients, resulting in fourfold downregulation of the IRF5 gene in lymphomas with 7q32 deletion versus deleted tumors.[33] Importantly, ectopic expression of IRF5 in marginal-zone lymphoma cells decreased proliferation and increased apoptosis in vitro, and impaired lymphoma development in vivo, suggesting that IRF5 may be a haploinsufficient tumor suppressor in this lymphoma entity.

Another layer of complexity in the pathogenesis of splenic MZL was revealed by the discovery of frequent microRNA dysregulation.[34] When compared to reactive lymphoid hyperplasia and other lymphoproliferative diseases, splenic MZL is found to have distinct expression of miRNAs, including highly overexpressed miR-155 and miR-34a, and repressed expression of miR-377 and miR-145, which are candidate diagnostic tools for splenic MZL.

Nodal MZL is a rare entity of MZL and is also associated with HCV infection, though probably to a lesser degree than splenic MZL. No characteristic translocations or chromosome imbalances, such as the t(11;18) in MALT lymphoma and del7q in splenic MZL, have been described in nodal MZL. Only a few cytogenetic alterations of NMZL have been reported, including trisomy 3 in 50% to 70% of cases, none with a clearly established role in either the diagnosis or pathogenesis of nodal MZL. Recent studies by Arribas and colleagues demonstrated that nodal MZL is characterized by upregulation of the IL6 and IL2 cytokine pathways and CD40 signaling, which are involved in B-cell

survival.[35] Some of the highly expressed genes include SYK, which is involved in BCR signaling, and TAC1, which is involved in activating NFκB. In the molecular signature of nodal MZL, a large number of overexpressed genes were associated with the NFκB signaling pathway, such as CD74, CD81, CD82, RELA, and TRAF4, reaffirming the central role of NFκB as a converging point for the proliferation and survival of MZL cells. Interestingly, some microRNA species known to be involved in memory B-cell development, including, for example, miR-221, miR-223, and let-7f, were overexpressed, suggesting their potential utility in the diagnosis of nodal MZL.

Our increased understanding of the biology of MZL in the past decade has already changed how we manage these patients. Because the infectious agents have been found to be required to initiate and maintain the malignant phenotype of some MZL subtypes, antimicrobial treatment has become a highly successful strategy in many patients. The majority of patients with *H. pylori*–associated MALT lymphoma can be cured with antibiotics that eliminate the bacterium, thus sparing a large number of patients more aggressive and expensive treatments.[36] Treatment of HCV and *C. psittaci* are effective strategies to manage patients with splenic MZL and MALT lymphoma of the ocular adnexa. The discovery of antigen stimulation and the central role of the BCR and NFκB signaling in MZL certainly supports the rationale to explore inhibitors of these pathways with Syk and BTK inhibitors, and even proteasome inhibitors. Targeting NOTCH2, which has been studied predominantly in ALL, could also be an effective strategy in the treatment of splenic MZL.

In contrast to MZL, CLL/SLL are diseases with clearly identified cytogenetic aberrations that have been shown to correlate with prognosis and survival. The clonal population of B lymphocytes that characterize CLL express CD19, CD5, and CD23, with often dim expression of CD20. In addition, these cells typically exhibit reduced levels of surface immunoglobulin. Collectively, this features are the hallmark of mature and activated B lymphocytes.[37] As with many of the other lymphoid malignancies, as our understanding of the pathogenesis of these diseases evolves, our appreciation of their heterogeneity has broadened. This heterogeneity seen in CLL can be characterized at numerous levels, including the status of mutation of the V genes, the expression of CD38, ZAP-70, and other discrete cytogenetic lesions.

For example, CLL cases can be subdivided on the basis of mutations in the V genes, based on the direct comparison of the DNA sequence of germline V genes with the V genes in the CLL cells.[38] CLL cases can then be classified as mutated (that is, they exhibit a more than 2% difference from the germline sequence) or unmutated. Expression of ZAP-70, a protein that is normally expressed near the surface of T cells known to play a role in signaling through the TCR, has also been shown to be an independent prognostic factor.[39-41] In CLL it has been found to be profoundly prognostic. Patients who are ZAP 70^- have been shown to have a median survival of 8 years, compared to patients who are ZAP-70^+, who have a median survival of approximately 25 years. Collectively, these factors have been used to risk stratify patients with CLL, such that those patients with B-CLL clones having few to no V-gene mutations, or with $CD38^+$ and ZAP-70^+ B-CLL, have been found to have comparatively more aggressive, usually fatal disease. In contrast, those patients with mutated V-genes and/or little to no CD38 or ZAP-70 are typically considered to have a very indolent natural history.[42]

Recurring cytogenetic lesions have also been described and may be more valuable in risk stratifying patients with CLL, and ultimately in developing better tailored therapies for the disease. One of the most common is deletion of 13q14.3, which occurs in more than 50% of cases over time.[43] This region of the chromosome is thought to encode two miRNA genes.[44] However, some of the highest risk cytogenetic lesions include deletions of 11q22-23, 17p13, and 6q21.[45] Although the specific genes involved in these deletions remain to be precisely identified, loss of 17p13 is thought to be associated with loss of the tumor suppressor p53 function, and deletion of 11q22-23 is thought to be associated with loss of the ataxia-telangiectasia mutated (ATM) gene. Specific treatment regimens for these discrete genetic subcategories of CLL are in development, and some principles may be emerging. First, patients with loss of 17p are thought to have a very poor prognosis and are typically very refractory to chemotherapy. Some anecdotes suggest that these patients may respond well to anti-CD52–based therapy (alemtuzumab), and it is thought that BTK inhibitors might override this adverse prognostic feature. Similarly, patients who have loss of the ATM function through deletion of 11q22-23 may benefit from the introduction of alkylating agents into the treatment program.

Pathogenesis of Mantle-Cell Lymphoma

Mantle-cell lymphoma (MCL) accounts for approximately 6% of all cases of non-Hodgkin lymphoma, or about 3000 cases per year in the United States.[1] Only a decade ago, it was primarily thought to be one of the most challenging subtypes of lymphoma to treat, carrying the incurable characteristics of indolent lymphoma, and the unfavorable features of aggressive lymphoma. Over the past decade, treatment paradigms for the disease have changed rapidly, and so too has the natural history. These changes are attributed to a

number of observations: (1) the recognition that MCL is not one disease and, like DLBCL, is a very heterogeneous disease composed of more indolent and aggressive variants; (2) the finding that intensive induction chemotherapy regimens consolidated by ASCT used in the front line can consistently produce a response rate of 90% to 100% and produce prolonged progression-free survival compared to traditional R-CHOP–based chemotherapy regimens; and (3) the emergence of novel drugs affecting unique targets, such as proteasome inhibitors and immunomodulatory drugs, which have led to new opportunities to either complement existing treatment paradigms or manage relapsed or refractory disease without specific cytotoxic therapy. What has become painfully clear over the past several years, however, is that given the heterogeneity of the disease, certainly at a biologic level, failure to characterize future populations of patients with MCL on clinical study in this regard will lead to confounding of conclusions about future treatments.

The molecular hallmark of MCL is overexpression of cyclin D1.[46] Although dysregulation of cyclin D1 has been recognized for some time, it has now become evident that MCL is a disease, possibly the prototypical disease, defined by gross cell cycle dysregulation. Cyclin D1 dysregulation is the pathognomonic chromosomal translocation t(11;14)(q13;q32) of the disease, which places cyclin D1 downstream of the highly active IgH enhancer.[47] Essential to the pathogenesis of the disease, the mRNA of cyclin D1 undergoes alternative splicing to produce two unique transcripts: cyclin D1a, which has been clearly shown to drive much of the cell cycle dysregulation; and cyclin D1b, whose expression is more variable and whose role is less well defined.[48] Deletion or mutation of the cyclin D1a mRNA tail region produces a truncated version of cyclin D1a mRNA, which has been shown to be 6 to 10 times more stable than the wild-type full-length cyclin D1a mRNA.[49] In addition to the pivotal role the splice variants of cyclin D1 mRNA play, microRNAs, specifically miRNA61, downregulate cyclin D1a, potentially modulating the cell cycle kinetics. The influence of miR61 can also be influenced by mutations in the tail region of the cyclin D1a mRNA in MCL, which can abrogate the miR61 binding site, allowing for the marked overproduction of cyclin D1 protein.[50] In addition, a number of important translational events have been shown to regulate cyclin D1 level. Phosphorylation of cyclin D1 by GSK3β leads to polyubiquitination by the E3 ligase FBX4, rendering it a substrate for the proteasome and proteolytic degradation.[51] Inactivation of GSK3β would preempt cyclin D1 phosphorylation, which has been shown to occur in the setting where AKT is aberrantly activated,[52] whereas impaired proteolytic degradation of cyclin D1 can be prevented by mutation of the E3 ligase FBX4,[53] resulting in reduced ubiquitination of the D1 protein. Collectively, these overlapping mechanisms of cyclin D1 regulation ensure high levels of cyclin D1 protein in MCL and, in select settings, can lead to marked impact on the cell cycle kinetics.

Although cyclin D1 levels are central in the pathogenesis of MCL, it is by no means the only corrupted cell cycle pathway.[54] Cyclin D1 normally interacts with CDK4/CDK6 to facilitate cell cycle progression through the G1-S checkpoint.[55] CDK4 is frequently overexpressed or amplified in MCL,[56] which would further limit the checkpoint control. Conversely, impaired inhibition of cell cycle regulation can play a major role in the disease, as evidenced by the fact that patients with MCL often have little to no accumulation of the CDK inhibitors p16 and p27.[57] Loss of p16 influence has been shown in patients with MCL—this tumor suppressor is deleted in about half of all MCL cases, and either mutated or silenced by promoter hypermethylation.[58] Impaired cell cycle control due to loss of p27 has been attributed to a number of mechanisms. The protein level of p27 in MCL is regulated by the ubiquitin proteasome. The p27-specific F-box protein Skp2 is inducible and been shown to be overexpressed in some patients with MCL, in particular those with disease that is known to be more aggressive. Increased levels of Skp2 lead to more active ubiquitin ligases, which leads to more prompt and efficient ubiquitination of p27 and thus a theoretically shorter half-life for the protein. This loss of cell cycle inhibition has been noted in patients with more aggressive forms of the disease.[59] This convergence of markedly dysregulated cell cycle control processes, involving both drivers and inhibitors of cell cycle kinetics, conspires to affect the proliferative rate of the disease and, as we now know, the risk stratification of select patient subpopulations.

The notion that the proliferative rate of the disease can be prognostic has now been confirmed at a number of levels. Patients with the truncated version of the mRNA of cyclin D1a, the stabler form, are known to have disease with a higher proliferative index[49] and more aggressive histologies, such as blastoid MCL.[60] Remarkably, the median overall survival (OS) of MCL patients with the truncated cyclin D1a mRNA is only 1.38 years, compared to 3.28 years for patients with the full-length and unstable mRNA.[49] The levels of the full-length mRNA transcript of cyclin D1a have been shown to positively correlate with the proliferation index as assayed by positive immunostaining of Ki-67. Ki-67 is a nuclear protein found in all states of the cell cycle (G_1, S, G_2, and M), but is absent from resting cells (G_0). Many cell cycle regulatory factors have been shown to correlate with the protein level of cyclin D1 on immunohistochemical staining, including the percentage of MCL cells with positive nuclear staining of the protein or the amount of nuclear staining of cyclin D1.[60] Conversely, the expression of p27 is inversely associated with Ki-67, where high levels of p27 appear to be

associated with lower proliferative index and improved overall survival.[57] These lines of data strongly support the contention that MCL is a disease grossly characterized by cell cycle dysregulation, and that these features of the underlying disease biology can be highly prognostic.

Further confirming the significance of the proliferative index, several studies have shown that nuclear staining of Ki-67, and using a cutoff of 30%, predicted OS, where patients below and above the 30% cutoff experienced a median OS of 13 months and 45 months, respectively.[61] Using yet another approach, a group at the National Cancer Institute (NCI) employed GEP to characterize patients with MCL (Figure 29-5). Their profiling experience firmly established that heterogeneity of the disease, which they used to define the proliferation signature of MCL. They discovered 48 genes whose expression levels were highly correlated with survival in a statistically significant manner. Among those 48 genes were a group of 20 genes that were variably expressed, though strongly correlated with cellular proliferation. They called the pattern of gene expression the "proliferation signature," which could essentially stratify patients along a continuum from highly proliferative to less proliferative disease.[62] Calculation of the average expression levels of these 20 genes in the proliferation signature allowed for the separation of the population into quartiles. The median OS of patients with the lowest expression of proliferation signature genes (quartile 1) was 6.71 years, whereas those with the highest expression (quartile 4) exhibited an OS of only 0.83 years. Surprisingly, cyclin D1 was not among the proliferation signature genes identified using the data generated from DNA microarrays, because the chip-based technology detected only the full-length mRNA of cyclin D1, which is unstable. Employing a reverse transcriptase (RT)-PCR assay to differentiate the two species of the cyclin D1 mRNA showed that patients with the truncated mRNA of cyclin D1 (3′ UTR low) demonstrated a higher expression level of cyclin D1, a higher expression of the 20 "proliferation signature" genes, and a substantially shorter OS, compared to patients with the full-length mRNA of cyclin D1 (i.e., 3′ UTR high).[62] When RT-PCR was designed to detect only

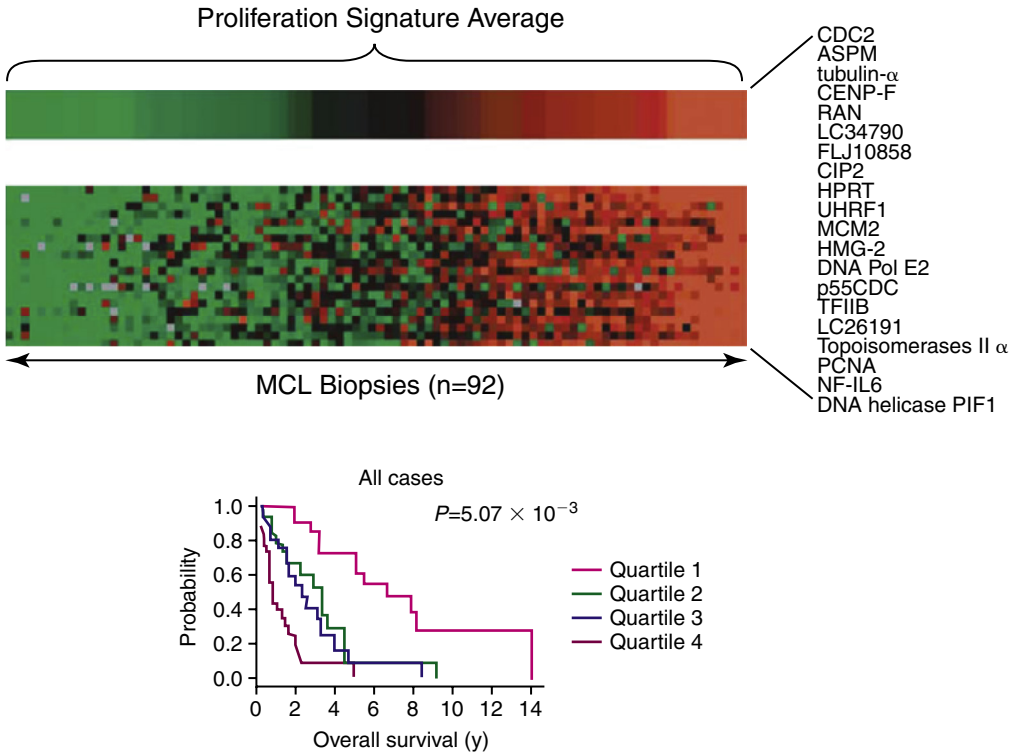


Figure 29-5 Gene expression profiling of mantle-cell lymphoma cases reveals prognostic significance of the proliferation index Gene expression profiling of primary mantle-cell lymphoma cases has demonstrated that the most aggressive forms of the disease are associated with an enrichment of genes involved in cell proliferation, a higher proliferative index, and a poorer prognosis. Cases with a lower proliferative index have a better prognosis. Proliferation gene expression signature may predict survival in MCL. These authors established a molecular diagnosis of MCL based on gene expression that can distinguish the disease from other lymphoma types. Using this diagnostic tool, they were able to investigate gene expression patterns in patient samples and evaluate whether there was a correlation with survival. In addition, they were able to investigate the genetic aberrations that are the basis of the pathobiology of MCL. This figure shows the proliferation signature average (PSA) for 92 samples from patients with MCL. The PSA is the average expression of the top 20 genes that were expressed most variably in MCL (i.e., the top third). To visualize the power of this as a predictive model, patients were ranked according to the expression of these "signature" genes in their tumors and then divided into four equal quartiles as shown. This analysis allows a suggested quantitative relationship between proliferation and survival. The Kaplan-Meier plots of overall survival of patients in each of these quartiles indicate that the proliferation signature can identify subgroups of patients with different survival times. Median survival times for each quartile were 6.71 years (1), 3.28 years (2), 2.31 years (3), and 0.83 years (4). *Data from Rosenwald A et al.* Cancer Cell. *2003;3:185-197.*

the coding region of cyclin D1 mRNA, which accounted for both the full-length and truncated mRNA, the levels of cyclin D1 were again shown to correlate with patient survival.

There is no question that the proliferative index of individual patients with MCL is prognostic. There is also no doubt that failing to characterize study populations with regard to this biologic parameter will lead to confounding influences in our interpretation of clinical trial data. The prognostic impact of the proliferation index remains relatively significant irrespective of the therapy, as had been shown with rituximab,[63] high-dose chemotherapy and stem cell transplant,[64] and the proteasome inhibitor bortezomib.[65] Moving forward, it will be critical to determine if the proliferation signature will prove useful in predicting responses to specific treatments, or even if it could be used to tailor treatment to a particular disease context. Although prognosticating patients with MCL using DNA microarray-based gene expression profiling remains an experimental approach, RT-PCR–based measurement of the expression of proliferation signature genes[62] may be validated and practical in the near future.

Pathogenesis of Lymphomas Derived from Thymic B cells: Primary Mediastinal Large B-Cell Lymphoma, Hodgkin Lymphoma, and Gray Zone Lymphomas

Lymphomas arising in the anterior mediastinum have posed significant diagnostic and management challenges over the past several decades, especially given the overlapping biologic and clinical features of these diseases. Emerging molecular data have begun to demonstrate important differences in the molecular pathogenesis of these diseases, especially the cell of origin. Both bone marrow stroma and thymic stromal cells express ligands and cytokines required for B-cell differentiation. Despite the shared roles marrow and thymic stroma play in B-cell differentiation, B-cell development in the thymus is very restricted. B-cell precursors are present in the thymus and are felt to be distinct from B-cell precursors in the bone marrow. Although the thymic microenvironment is the source of lymphopoietic factors that include interleukin-7 (IL-7), pro-B cells exposed to the thymic microenvironment are hyporesponsive to IL-7, whereas pro-B cells derived from the bone marrow are typically responsive. Thus, pro-B cells from the thymus accumulate at an early pro–B-cell stage of development, cycle less than their bone marrow counterparts, and fail to differentiate efficiently.[66] This difference in the microenvironment and its effects on the maturation of B cells may account for the unique features for lymphomas that arise from thymic B cells compared to lymphomas arising from the bone marrow and other lymphoid tissue.

Based merely on histopathologic features, including the expression of CD20, primary mediastinal large B-cell lymphoma (PMBL) had always been considered a diffuse large B-cell lymphoma (DLBCL), at least until it was recognized as a discrete entity in the REAL Classification in 1994.[67] Despite the histopathologic similarities with DLBCL, it was becoming evident in the early 1980s that the clinical presentation of PMBL and DLBCL, as well as the prognosis, was significantly different. Differences in the molecular pathogenesis of these diseases became evident when differences in gene expression profiling demonstrated extensive overlap between PMBL and classical Hodgkin lymphoma–nodular sclerosing (cHL-NS)[68] (Figure 29-6). Though unclear for years, it is now widely recognized that the Hodgkin–Reed Sternberg (HRS) cells are characterized by suppression of many components of the B-cell program, and they are incapable of immunoglobulin secretion.[69] Efforts to identify the differences between PMBL and other subtypes of B-cell lymphoma using gene expression profiling led to the surprising observation that PMBL was strikingly similar to the profiles seen for cHL rather than for other subtypes of DLBCL. In a study by Rosenwald and colleagues, more than one third of the genes that were more highly expressed in PMBL compared to DLBCLs were also characteristically expressed in Hodgkin lymphoma cells.[68] In this study, the PDL2 gene, which encodes a regulator of T-cell activation, was the gene that best discriminated PMBL from other DLBCLs. PDL2 was more highly expressed in PMBL and cHL and either not expressed or expressed to minor levels in DLBCL. The genomic loci for PDL2 and several neighboring genes were amplified in more than half of cases of PMBL and Hodgkin lymphoma and expressed to much lower levels in DLBCL. Collectively, these data gave credence to the observation that B-cell lymphomas derived from the thymus were a unique entity based on the shared pathogenetic features and likely clinical history. It also established that they were markedly different compared to those B-cell lymphomas derived from the bone marrow compartment.

Genetic studies of PMBL frequently demonstrate amplification of recurring regions on chromosome 9p and 2p, which, interestingly, have also been described in cHL, and rarely in other subtypes of DLBCL.[70] The 9p region encodes JAK2, a tyrosine kinase that phosphorylates and activates the transcription factor STAT6. SOCS1, which suppresses JAK signaling, is regularly deleted in both PMBL and cHL, contributing to further dysregulation of the JAK-STAT pathway.[71] Other genes thought to be involved at 9p include PDL1 and PDL2, whereas c-Rel may be involved at 2p. Like cHL, PMBL also has constitutively activated NFκB and loss of CIITA (MHC 2), which may help explain

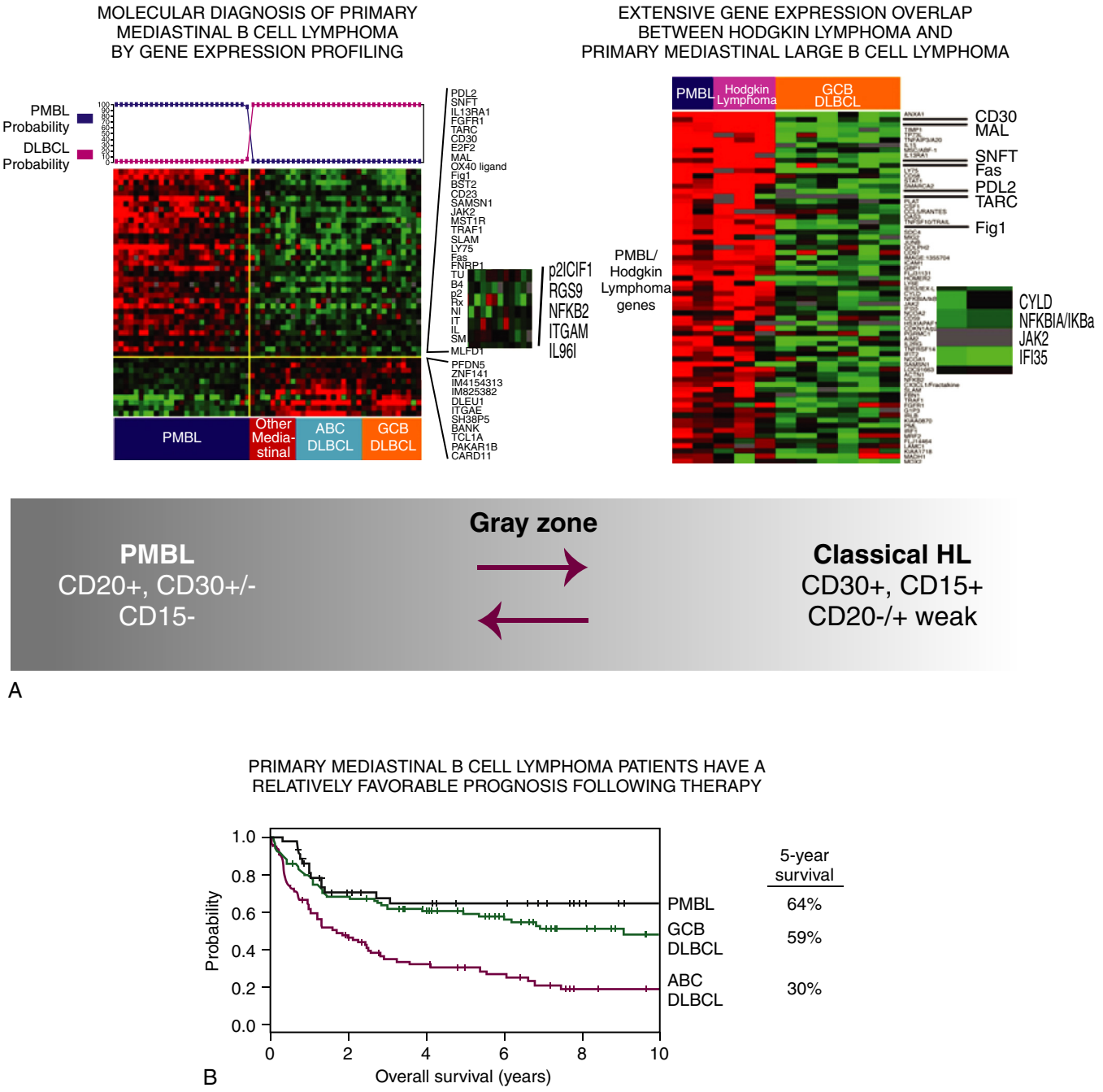


FIGURE 29-6 **(A)** Gene expression profiling of primary mediastinal B-cell and Hodgkin lymphoma reveal marked similarities with each other, and marked dissimilarity with DLBCL. **(B)** Patients with PMBL have a prognosis similar to that for Hodgkin lymphoma, and superior to that for patients with DLBCL.

the ability of these cells to evade immune surveillance.[72] Although PMBCL and cHL share a cell of origin in the form of the thymic B-cell, as well as molecular characteristics, there are still important morphologic and immunophenotypic distinguishing features between these two diseases with significant clinical and treatment implications.

Another example of a thymic B-cell lymphoma, recognized only in 2008 by the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues, is the entity known as *gray zone lymphomas* (GZL), now more widely referred to as *B-cell lymphoma* with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma.[4] GZL represents a mediastinal B-cell lymphoma that does not fulfill the morphologic and/or phenotypic criteria for PMBL or CHL, but instead represents a spectrum between these two entities.

Interphase FISH studies of GZL have demonstrated a number of recurring molecule lesions, including amplification of the REL/BCL11A locus, alterations affecting the JAK2/PDL2 locus, and rearrangement of the CIITA (MHC2)

locus, as well as gains of MYC.[73] Although the molecular signature of GZL has not been elucidated, a recent large-scale methylation analysis of PMBL, CHL, and GZL has demonstrated a number of similarities in the methylation profile of these diseases, although GZL did exhibit its own unique profile distinct from PMBL and cHL.[74] Importantly, GZL could be distinguished from cHL-NS and PMBL by differential methylation of selected CpG islands, and a class prediction model could be established to segregate the various entities. Thus, the global methylation profile of these entities may be useful not only to establish new diagnostic tools and clarify the pathogenesis of these lymphomas, but also to identify possible targets for future therapies.

While lymphomas of the thymic B-cell origin (cHL-NS, GZL, and PMBL) are closely related diseases that share clinical and biologic characteristics, there remains a spectrum of heterogeneity.

As the field of medical oncology enters the molecular and targeted therapy era, the biology that connects this disease and sets some lymphomas apart from DLBCL can pave the way for new therapeutic platforms that optimize efficacy and limit toxicity. The elucidation of pathways and molecular targets that are unique to the thymic B-cell lymphoma group of disease gives credence to therapies targeting NFκB, Janus kinases (JAK), programmed death-1 (PD-1), and epigenetic-based therapies.

Molecular Pathogenesis of the Peripheral T-Cell Lymphomas

The mature T-cell lymphomas, also referred to as *peripheral T-cell lymphomas* (PTCL), arise from thymic T cells or NK cells. These diseases are associated with often diverse presentation, arising in either the nodal or extranodal compartments.[4] Molecular and genetic characterization of PTCLs has lagged well behind that of B-cell non-Hodgkin lymphomas—in part because of their rarity and the relatively specific morphologic and immunophenotypic features of these neoplasms, which has precluded reliable identification of the cell of origin and classification into distinct disease subtypes.

Studies using conventional cytogenetic analysis over the past two decades have revealed only limited, disease-specific, recurrent karyotypic abnormalities.[75] CGH studies have helped delineate genetic differences, as well as similarities, between different subtypes of PTCL.[76] Gene expression profile analyses are providing insights into cell-intrinsic and microenvironment-related pathogenetic features in disease subsets,[77] as well as genetic signatures associated with clinical outcomes.[77] Higher resolution genetic analyses, specifically exome and genome sequencing using next-generation techniques, might help refine diagnostic categories and provide prognostic and therapeutically actionable information.

Recent advances in immunophenotypic analysis and systematic clinical characterization of large series of PTCL have led to the recognition of more than 22 different defined or provisional clinicopathologic subtypes of T-cell lymphoma that are distributed among four different subcategories in the present WHO classification (2008).[4] Molecular and genetic aspects of disease pathogenesis of some of the more common and better defined subtypes of PTCL are described here.

Anaplastic Large-Cell Lymphoma (ALCL)

Two different types of ALCLs are presently recognized, including ALK$^+$ ALCL and ALK$^-$ ALCL, the former associated with a superior outcome relative to ALK$^-$ ALCL. Although these entities share similar morphologic and immunophenotypic features, there are a number of key differences in their biologic and clinical characteristics. ALK$^+$ ALCL is characterized by the chromosome translocation t(2;5)(p23;q35) in 55% to 85% of systemic ALCL, or variant translocations involving ALK and other partner genes in a minority of cases.[78,79] The translocation t(2;5)(p23;q35) results in a fusion protein, nucleophosmin (NPM)/ALK (NPM-ALK), which leads to the constitutive activation of the ALK tyrosine kinase and alterations in multiple signaling pathways, including the Janus kinase 3 (JAK3)/signal transducer and activator of transcription (STAT3), phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase (AKT)/mammalian target of rapamycin (mTOR), and the phospholipase C-γ (PLC-γ) mediated RAS-extracellular signal–regulated kinase (ERK) pathways.[78] Activation of Notch1 signaling by its ligand Jagged1, which is expressed on neoplastic and neoplastic cells, has also been proposed to play a role in the pathogenesis of ALK$^+$ ALCL.[80] Secondary MYC translocations have been reported in some cases with an aggressive behavior.[81]

It is unclear at present whether ALK$^+$ ALCL originates from cytotoxic T cells or acquires a cytotoxic phenotype, but studies have reported activation of a Th17 differentiation program in this disease.[77] Molecular alterations in signaling pathways as a consequence of the variant translocations are not adequately understood, but similarities and differences in gene expression profiles with NPM-ALK have been described.[82] Differences in gene expression profiles between certain morphologic subtypes of ALK$^+$ ALCL have also been reported,[83] but array CGH analysis of NPM-ALK and variant ALK translocations associated with ALCL have revealed similar recurrent secondary genetic abnormalities.[76]

The cell of origin of ALK⁻ ALCL is also not known at present. Array CGH analysis of ALK⁺ and ALK⁻ ALCL has highlighted differences in secondary genetic aberrations between the two subtypes,[76] and differential expression of microRNAs has also been reported.[84] Comparative gene expression analysis of ALK⁺ and ALK⁻ ALCL has revealed deregulation of common kinase signaling cascades and regulators of apoptosis, as well as differences between these subtypes, such as alterations in cell cycle regulators, signal transduction proteins, and various transcription factors.[85] ALK⁺ ALCL shows overexpression of genes implicated in immune or inflammatory responses, regulation of NFκB signaling, and lymphocyte migration and adhesion, whereas ALK⁻ ALCL exhibits overexpression of genes involved in certain cytokine signaling pathways.[83] Analysis of ALK⁻ ALCL using next-generation massively parallel sequencing has revealed a recurrent balanced translocation t(6;7)(p25.3;q32.3), leading to the juxtaposition of the DUSP22 phosphatase gene with the fragile site FRA7H, which results in the downregulation of DUSP22 and upregulation of MIR29 microRNAs located on 7q32.3.[86] This translocation was observed in 45% of ALK⁻ ALCLs with 6p25.3 rearrangements and in both systemic and cutaneous forms of ALK⁻ ALCL. The biologic consequence of this translocation awaits further investigation.

Peripheral T-Cell Lymphoma Not Otherwise Specified

PTCL-NOS is the most common subtype of PTCL, accounting for up to 25% of all PTCL occurring worldwide. It is a clinically heterogeneous entity, potentially comprising different PTCL subtypes at different stages of disease evolution. Comparison of PTCL-NOS expression profiles with those of purified T-cell subsets has suggested a relationship between PTCL-NOS subtypes with either activated CD4⁺ or CD8⁺ T cells,[87] and cases exhibiting a gene expression profile similar to cytotoxic T cells have been shown to have inferior survival.[77] PTCL-NOS lacks specific, recurrent cytogenetic abnormalities, but complex cytogenetic aberrations have been associated with a poor prognosis in PTCL-NOS.[75] Recently, the translocation t(5:9)(q33:32) was reported in 17% of PTCL-NOS, which results in the fusion of the *IL-2 inducible T-cell kinase* (ITK) gene with the *spleen tyrosine kinase* (SYK) gene.[88] Transgenic mice expressing the ITK-SYK fusion transcript develop T-cell lymphomas mimicking the human disease.[89,90] Overexpression of Syk tyrosine kinase and Syk phosphorylation, in the absence of *SYK* translocations, has also been observed in PTCL.[86]

Based on the expression levels of the NFκB pathway genes, PTCL-NOS can be segregated into two groups showing differences in survival.[91] The differential expression of a set of proliferation and cell cycle–associated genes, including CCNA, CCNB, TOP2A, and PCNA, has been shown to predict prognosis.[92] PTCL-NOS shows deregulation of pathways controlling apoptosis, cell proliferation, adhesion, matrix remodeling, and chemoresistance, and upregulation of platelet-derived growth factor receptor alpha has been observed in many cases.[87]

Distinguishing PTCL-NOS from ALK⁻ ALCL can be difficult using current cytomorphologic and immunophenotypic criteria. This is mirrored at the chromosomal and genetic level. Early gene expression profiling studies were unable to distinguish PTCL-NOS from other PTCL subtypes.[91] However, analysis restricted to nodal PTCL allowed discrimination between PTCL-NOS and other PTCL subtypes and further subclassification based on alterations of different biologic processes or signaling pathways.[9] Refined, supervised gene expression profile analysis can provide a signature allowing distinction between PTCL-NOS and ALK⁻ ALCL; however, the pathogenetic significance of the involved genes and pathways is not yet clear.[94] Recently a model comprising three genes, TNFRSF8, BATF3, and TMOD1, obtained from a meta-analysis of the transcriptional profiles of a large series of PTCL, was shown to distinguish ALK⁻ ALCL from PTCL-NOS.[95] Future studies might illuminate the biologic basis of this observation. PTCL-NOS and ALK⁻ ALCL also share karyotypic abnormalities, including gains of chromosomes 1q and 3p and losses on chromosome 6q, although the loci on 6q have been shown to differ.[75] CGH analysis has shown overlapping aberrations, including 6q and 13q losses, as well as subtype-specific abnormalities.[96] Recurrent chromosome gains of 7q that target cyclin-dependent kinase 6[97] and 8q involving the *MYC* locus[98] have been reported in PTCL-NOS. A recent genome-wide NGS analysis of PTCL led to the identification of recurrent translocations involving p53-related genes, including rearrangements of the *TP63* gene with TBL1XR1 and ATXN1 genes.[99] These gene fusions encode proteins that inhibit the p53 pathway and are associated with adverse clinical outcomes. Screening a large series of cases for *TP63* rearrangements by FISH showed similar frequencies in PTCL-NOS (9.4%), ALK⁻ ALCL (12.5%), and primary cutaneous ALCL (10.5%).

Angioimmunoblastic T-Cell Lymphoma

AITL is thought to derive from T-follicular helper (TFH) cells based on phenotypic features and overexpression of genes characteristic of normal TFH cells (CXCL13, BCL6, PD-1, CD40LG, and NFATC1).[100,101] Microenvironmental factors and signals orchestrating tumor-stroma cross talk are

thought to play a role in AITL pathogenesis. Recent studies, however, have also demonstrated a TFH phenotype for a subset of PTCL-NOS.[102] This might be one of the explanations for the inability of gene expression profiling to segregate AITL from subsets of PTCL-NOS in some instances.[87] Gains of chromosomes 3q, 5q, and 21 have been described as recurrent alterations in AITL, although the genes affected by these changes are not known.[75] Studies from transgenic mouse models and genome sequencing studies have begun to provide insights into AITL pathogenesis and its relationship with other types of PTCL. Mice engineered to disrupt the function of the *Tet2* gene showed an increase in T-cell progenitors and developed T-cell lymphomas. On sequencing human lymphoma specimens, heterozygous insertions and deletions of *Tet2* were detected in 33% of AITL and smaller subsets of other PTCLs.[103] Analysis of a large cohort of PTCL revealed a higher frequency of *Tet2* mutations in AITL (47%) and PTCL-NOS (38%) and an association with adverse clinical features.[104] Of interest, PTCL-NOS expressing TFH markers, despite showing or lacking a histopathologic resemblance to AITL, demonstrated a significantly higher frequency of *Tet2* mutations. Furthermore, *DNMT3A* mutations occur in a high frequency (73%) of cases harboring *TET2*, including PTCL-NOS and AITL, suggesting oncogenic cooperation between pathways regulated by *TET2* and *DNMT3A* such as cytosine methylation and demethylation processes in PTCL.[105] Recently, heterozygous IDH2 mutations, mostly resulting in an R172 substitution, have been described in 20% to 45% of AITL.[106] The prognostic implications of this mutation, if any, are unclear at present. It also remains to be seen whether this mutation is specific for AITL or other subtypes of PTCL derived from TFH cells. Overall, these studies indicate an important contribution of epigenetic alterations in the pathogenesis of AITL and at least a subset of PTCL-NOS.

Translating Molecular Pathogenesis into Novel Treatment Platforms

One of the many objectives for improving our understanding of the molecular basis of any disease is ultimately to use that information to treat disease in a more biologically rational manner. The explosion of detailed mechanistic studies into the pathogenesis of lymphoma has begun to create new opportunities for therapeutic intervention. Although we could point to many studies in various stages of development, the focus here is on one in ABC-DLBCL.

As discussed earlier, the emergence of new small molecules targeting BTK has provided an immediate opportunity to treat B-cell lymphomas at a fundamental level. Enrichment for dysregulated BCR signaling in ABC-DLBCL offers an opportunity to improve the outcome of a subset of DLBCL associated with an inferior prognosis. The recent development of the BTK inhibitor ibrutinib has been shown to completely block BCR signaling and induce apoptosis. Ibrutinib forms a bond with cysteine-148 in BTK, inhibiting the kinase with an IC50 of 0.5 nM, and with a relatively high degree of specificity. In a recent study reported by the NCI, 49 patients with relapsed or refractory DLBCL derived from the GC (n = 20) or ABC origin (n = 29) were treated with ibrutinib at the maximum tolerated dose. These patients were overall very heavily treated, with a median of three to four prior therapies, with a substantial number having refractory disease. With the caveats of a small unrandomized study, the overall response rates were 41% among those patients with ABC DLBCL (12 of 29 responding), compared to 5% (1 of 20) among those patients with GC-DLBCL. In addition, although no complete remissions were seen in the GC group, 17% of the responses seen in the ABC subtype were complete remissions. When the response in the ABC group was characterized as a function of CD70b and MYD88 mutation, the response rates seen among patients with mutant CD79b, wild-type CD79b, and mutant 79b/MYD88 were 71% (5 of 7), 34% (10 of 29), and 80% (4 of 5), further supporting the idea that drugs targeting specific pathways known to be dysregulated in a particular biologic subset of disease may be able to help overcome some of the adverse prognostic features of that disease.

Future Directions

Our rapidly evolving understanding of lymphoma at the molecular level has afforded new opportunities to better risk stratify patients, which will likely lead to more reasonable tailoring of treatment, and has firmly created the opportunity to more precisely treat these diseases with respect to their underlying biologic basis. At present, small molecules targeting BCR signaling mechanisms, PI3-kinase, Bcl-2 family members, and a host of monoclonal antibodies and antibody drug conjugates offer the potential to tailor the use of these small molecules with complementing current treatment paradigms, or creating new ones with less of an emphasis on specific cytotoxic therapy.

Key References

1. Li A, et al. Utilization of Ig heavy chain variable, diversity, and joining gene segments in children with B-lineage acute lymphoblastic leukemia: implications for the mechanisms of VDJ recombination and for pathogenesis. *Blood*. 2004;103:4602-4609.
2. Peled JU, et al. The biochemistry of somatic hypermutation. *Annu Rev Immunol*. 2008;26:481-511.
3. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol*. 2006;6:127-135.
4. Swerdlow SH. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: International Agency for Research on Cancer; 2008.
5. Menon MP, Pittaluga S, Jaffe ES. The histological and biological spectrum of diffuse large B-cell lymphoma in the World Health Organization classification. *Cancer J*. 2012;18:411-420.
6. Choi WW, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res*. 2009;15:5494-5502.
7. Kohlmann A, et al. Next-generation sequencing—feasibility and practicality in haematology. *Br J Haematol*. 2013;160:736-753.
9. Sekiguchi N, et al. Follicular lymphoma subgrouping by fluorescence in situ hybridization analysis. *Cancer Sci*. 2005;96:77-82.
10. van Dongen JJ, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17:2257-2317.
11. Gu K, Chan WC, Hawley RC. Practical detection of t(14;18)(IgH/BCL2) in follicular lymphoma. *Arch Pathol Lab Med*. 2008;132:1355-1361.
12. Leich E, et al. Follicular lymphomas with and without translocation t(14;18) differ in gene expression profiles and genetic alterations. *Blood*. 2009;114:826-834.
14. Schmitt C, et al. The bcl-2/IgH rearrangement in a population of 204 healthy individuals: occurrence, age and gender distribution, breakpoints, and detection method validity. *Leuk Res*. 2006;30:745-750.
15. Katzenberger T, et al. Cytogenetic alterations affecting BCL6 are predominantly found in follicular lymphomas grade 3B with a diffuse large B-cell component. *Am J Pathol*. 2004;165:481-490.
16. Hartmann EM, Ott G, Rosenwald A. Molecular biology and genetics of lymphomas. *Hematol Oncol Clin North Am*. 2008;22:807-823, vii.
17. Voorhees PM, et al. Follicular lymphoma with a Burkitt translocation—predictor of an aggressive clinical course: a case report and review of the literature. *Arch Pathol Lab Med*. 2004;128:210-213.
18. Schwaenen C, et al. Microarray-based genomic profiling reveals novel genomic aberrations in follicular lymphoma which associate with patient survival and gene expression status. *Genes Chromosomes Cancer*. 2009;48:39-54.
19. Slack GW, Gascoyne RD. Next-generation sequencing discoveries in lymphoma. *Adv Anat Pathol*. 2013;20:110-116.
22. Thome M. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol*. 2004;4:348-359.
23. Kwee I, et al. Genomic profiles of MALT lymphomas: variability across anatomical sites. *Haematologica*. 2011;96:1064-1066.
24. Levy M, et al. Prognostic value of translocation t(11;18) in tumoral response of low-grade gastric lymphoma of mucosa-associated lymphoid tissue type to oral chemotherapy. *J Clin Oncol*. 2005;23:5061-5066.
25. Novak U, et al. The NFκB negative regulator TNFAIP3 (A20) is inactivated by somatic mutations and genomic deletions in marginal zone lymphomas. *Blood*. 2009;113:4918-4921.
29. Salido M, et al. Cytogenetic aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell lymphomas: a multicenter study of the Splenic B-Cell Lymphoma Group. *Blood*. 2010;116:1479-1488.
30. Rossi D, et al. The coding genome of splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating marginal zone development. *J Exp Med*. 2012;209:1537-1551.
31. Mensah AA, et al. Absence of NOTCH1 gene mutations in MALT lymphomas. *Br J Haematol*. 2012;157:382-384.
32. Watkins AJ, et al. An integrated genomic and expression analysis of 7q deletion in splenic marginal zone lymphoma. *PLoS One*. 2012;7:e44997.
33. Fresquet V, et al. High-throughput sequencing analysis of the chromosome 7q32 deletion reveals IRF5 as a potential tumour suppressor in splenic marginal-zone lymphoma. *Br J Haematol*. 2012;158:712-726.
34. Arribas AJ, et al. Splenic marginal zone lymphoma: comprehensive analysis of gene expression and miRNA profiling. *Mod Pathol*. 2013;26:889-901.
35. Arribas AJ, et al. Nodal marginal zone lymphoma: gene expression and miRNA profiling identify diagnostic markers and potential therapeutic targets. *Blood*. 2012;119:e9-e21.
36. Nakamura S, et al. Long-term clinical outcome of gastric MALT lymphoma after eradication of *Helicobacter pylori*: a multicentre cohort follow-up study of 420 patients in Japan. *Gut*. 2012;61:507-513.
39. Crespo M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348:1764-1775.
41. Wiestner A, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*. 2003;101:4944-4951.
46. Perez-Galan P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. *Blood*. 2011;117:26-38.
48. Marzec M, et al. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. *Blood*. 2006;108:1744-1750.
49. Wiestner A, et al. Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. *Blood*. 2007;109:4599-4606.
50. Chen RW, et al. Truncation in CCND1 mRNA alters miR-16-1 regulation in mantle cell lymphoma. *Blood*. 2008;112:822-829.
52. Dal Col J, et al. Distinct functional significance of Akt and mTOR constitutive activation in mantle cell lymphoma. *Blood*. 2008;111:5142-5151.
53. Barbash O, et al. Mutations in Fbx4 inhibit dimerization of the SCF(Fbx4) ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell*. 2008;14:68-78.
59. Lwin T, et al. Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas. *Blood*. 2007;110:1631-1638.
60. Shakir R, Ngo N, Naresh KN. Correlation of cyclin D1 transcript levels, transcript type and protein expression with proliferation and histology among mantle cell lymphoma. *J Clin Pathol*. 2008;61:920-927.
62. Rosenwald A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell*. 2003;3:185-197.
63. Determann O, et al. Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood*. 2008;111:2385-2387.
64. Garcia M, et al. Proliferation predicts failure-free survival in mantle cell lymphoma patients treated with rituximab plus hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with rituximab plus high-dose methotrexate and cytarabine. *Cancer*. 2009;115:1041-1048.
65. Goy A, et al. Potential biomarkers of bortezomib activity in mantle cell lymphoma from the phase 2 PINNACLE trial. *Leuk Lymphoma*. 2010;51:1269-1277.
68. Rosenwald A, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med*. 2003;198:851-862.
69. Schmitz R, et al. Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol*. 2009;4:151-174.
70. Green MR, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. 2010;116:3268-3277.

71. Melzner I, et al. Biallelic mutation of SOCS-1 impairs JAK2 degradation and sustains phospho-JAK2 action in the MedB-1 mediastinal lymphoma line. *Blood*. 2005;105:2535-2542.
72. Steidl C, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471:377-381.
73. Eberle FC, et al. Gray zone lymphoma: chromosomal aberrations with immunophenotypic and clinical correlations. *Mod Pathol*. 2011;24:1586-1597.
74. Eberle FC, et al. Methylation profiling of mediastinal gray zone lymphoma reveals a distinctive signature with elements shared by classical Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. *Haematologica*. 2011;96:558-566.
75. Nelson M, et al. Cytogenetic abnormalities and clinical correlations in peripheral T-cell lymphoma. *Br J Haematol*. 2008;141:461-469.
76. Salaverria I, et al. Genomic profiling reveals different genetic aberrations in systemic ALK-positive and ALK-negative anaplastic large cell lymphomas. *Br J Haematol*. 2008;140:516-526.
77. Iqbal J, et al. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood*. 2010;115:1026-1036.
78. Amin HM, Lai R. Pathobiology of ALK+ anaplastic large-cell lymphoma. *Blood*. 2007;110:2259-2267.
79. Stein H, et al. CD30$^+$ anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. *Blood*. 2000;96:3681-3695.
81. Moritake H, et al. C-MYC rearrangement may induce an aggressive phenotype in anaplastic lymphoma kinase positive anaplastic large cell lymphoma: identification of a novel fusion gene ALO17/C-MYC. *Am J Hematol*. 2011;86:75-78.
82. Bohling SD, et al. Analysis of gene expression profile of TPM3-ALK positive anaplastic large cell lymphoma reveals overlapping and unique patterns with that of NPM-ALK positive anaplastic large cell lymphoma. *Leuk Res*. 2008;32:383-393.
83. Lamant L, et al. Gene-expression profiling of systemic anaplastic large-cell lymphoma reveals differences based on ALK status and two distinct morphologic ALK+ subtypes. *Blood*. 2007;109:2156-2164.
84. Merkel O, et al. Identification of differential and functionally active miRNAs in both anaplastic lymphoma kinase (ALK)+ and ALK- anaplastic large-cell lymphoma. *Proc Natl Acad Sci U S A*. 2010;107:16228-16233.
85. Thompson MA, et al. Differential gene expression in anaplastic lymphoma kinase-positive and anaplastic lymphoma kinase-negative anaplastic large cell lymphomas. *Hum Pathol*. 2005;36:494-504.
86. Feldman AL, et al. Overexpression of Syk tyrosine kinase in peripheral T-cell lymphomas. *Leukemia*. 2008;22:1139-1143.
87. Piccaluga PP, et al. Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets. *J Clin Invest*. 2007;117:823-834.
88. Streubel B, et al. Novel t(5;9)(q33;q22) fuses ITK to SYK in unspecified peripheral T-cell lymphoma. *Leukemia*. 2006;20:313-318.
89. Pechloff K, et al. The fusion kinase ITK-SYK mimics a T cell receptor signal and drives oncogenesis in conditional mouse models of peripheral T cell lymphoma. *J Exp Med*. 2010;207:1031-1044.
90. Dierks C, et al. The ITK-SYK fusion oncogene induces a T-cell lymphoproliferative disease in mice mimicking human disease. *Cancer Res*. 2010;70:6193-6204.
91. Martinez-Delgado B, et al. Expression profiling of T-cell lymphomas differentiates peripheral and lymphoblastic lymphomas and defines survival related genes. *Clin Cancer Res*. 2004;10:4971-4982.
92. Cuadros M, et al. Identification of a proliferation signature related to survival in nodal peripheral T-cell lymphomas. *J Clin Oncol*. 2007;25:3321-3329.
93. Ballester B, et al. Gene expression profiling identifies molecular subgroups among nodal peripheral T-cell lymphomas. *Oncogene*. 2006;25:1560-1570.
94. Piva R, et al. Gene expression profiling uncovers molecular classifiers for the recognition of anaplastic large-cell lymphoma within peripheral T-cell neoplasms. *J Clin Oncol*. 2010;28:1583-1590.
95. Agnelli L, et al. Identification of a 3-gene model as a powerful diagnostic tool for the recognition of ALK-negative anaplastic large-cell lymphoma. *Blood*. 2012;120:1274-1281.
96. Zettl A, et al. Genomic profiling of peripheral T-cell lymphoma, unspecified, and anaplastic large T-cell lymphoma delineates novel recurrent chromosomal alterations. *Am J Pathol*. 2004;164:1837-1848.
97. Nagel S, et al. Amplification at 7q22 targets cyclin-dependent kinase 6 in T-cell lymphoma. *Leukemia*. 2008;22:387-392.
98. Thorns C, et al. Chromosomal aberrations in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma unspecified: a matrix-based CGH approach. *Genes Chromosomes Cancer*. 2007;46:37-44.
99. Vasmatzis G, et al. Genome-wide analysis reveals recurrent structural abnormalities of TP63 and other p53-related genes in peripheral T-cell lymphomas. *Blood*. 2012;120:2280-2289.
100. de Leval L, Gisselbrecht C, Gaulard P. Advances in the understanding and management of angioimmunoblastic T-cell lymphoma. *Br J Haematol*. 2010;148:673-689.
101. de Leval L, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood*. 2007;109:4952-4963.
102. Rodriguez-Pinilla SM, et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol*. 2008;32:1787-1799.
103. Quivoron C, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell*. 2011;20:25-38.
104. Lemonnier F, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood*. 2012;120:1466-1469.
105. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. *N Engl J Med*. 2012;366:95-96.
106. Cairns RA, et al. IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. *Blood*. 2012;119:1901-1903.

References

1. Li A, et al. Utilization of Ig heavy chain variable, diversity, and joining gene segments in children with B-lineage acute lymphoblastic leukemia: implications for the mechanisms of VDJ recombination and for pathogenesis. *Blood*. 2004;103:4602-4609.
2. Peled JU, et al. The biochemistry of somatic hypermutation. *Annu Rev Immunol*. 2008;26:481-511.
3. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol*. 2006;6:127-135.
4. Swerdlow SH. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: International Agency for Research on Cancer; 2008.
5. Menon MP, Pittaluga S, Jaffe ES. The histological and biological spectrum of diffuse large B-cell lymphoma in the World Health Organization classification. *Cancer J*. 2012;18:411-420.
6. Choi WW, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res*. 2009;15:5494-5502.
7. Kohlmann A, et al. Next-generation sequencing—feasibility and practicality in haematology. *Br J Haematol*. 2013;160:736-753.
8. Buchonnet G, et al. Distribution of BCL2 breakpoints in follicular lymphoma and correlation with clinical features: specific subtypes or same disease? *Leukemia*. 2002;16:1852-1856.
9. Sekiguchi N, et al. Follicular lymphoma subgrouping by fluorescence in situ hybridization analysis. *Cancer Sci*. 2005;96:77-82.
10. van Dongen JJ, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17:2257-2317.
11. Gu K, Chan WC, Hawley RC. Practical detection of t(14;18)(IgH/BCL2) in follicular lymphoma. *Arch Pathol Lab Med*. 2008;132:1355-1361.
12. Leich E, et al. Follicular lymphomas with and without translocation t(14;18) differ in gene expression profiles and genetic alterations. *Blood*. 2009;114:826-834.
13. Ott G, et al. Cytomorphologic, immunohistochemical, and cytogenetic profiles of follicular lymphoma: 2 types of follicular lymphoma grade 3. *Blood*. 2002;99:3806-3812.
14. Schmitt C, et al. The bcl-2/IgH rearrangement in a population of 204 healthy individuals: occurrence, age and gender distribution, breakpoints, and detection method validity. *Leuk Res*. 2006;30:745-750.
15. Katzenberger T, et al. Cytogenetic alterations affecting BCL6 are predominantly found in follicular lymphomas grade 3B with a diffuse large B-cell component. *Am J Pathol*. 2004;165:481-490.
16. Hartmann EM, Ott G, Rosenwald A. Molecular biology and genetics of lymphomas. *Hematol Oncol Clin North Am*. 2008;22:807-823, vii.
17. Voorhees PM, et al. Follicular lymphoma with a Burkitt translocation—predictor of an aggressive clinical course: a case report and review of the literature. *Arch Pathol Lab Med*. 2004;128:210-213.
18. Schwaenen C, et al. Microarray-based genomic profiling reveals novel genomic aberrations in follicular lymphoma which associate with patient survival and gene expression status. *Genes Chromosomes Cancer*. 2009;48:39-54.
19. Slack GW, Gascoyne RD. Next-generation sequencing discoveries in lymphoma. *Adv Anat Pathol*. 2013;20:110-116.
20. Parsonnet J, et al. *Helicobacter pylori* infection and gastric lymphoma. *N Engl J Med*. 1994;330:1267-1271.
21. Dierlamm J, et al. Characteristic pattern of chromosomal gains and losses in marginal zone B cell lymphoma detected by comparative genomic hybridization. *Leukemia*. 1997;11:747-758.
22. Thome M. CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol*. 2004;4:348-359.
23. Kwee I, et al. Genomic profiles of MALT lymphomas: variability across anatomical sites. *Haematologica*. 2011;96:1064-1066.
24. Levy M, et al. Prognostic value of translocation t(11;18) in tumoral response of low-grade gastric lymphoma of mucosa-associated lymphoid tissue type to oral chemotherapy. *J Clin Oncol*. 2005;23:5061-5066.
25. Novak U, et al. The NFκB negative regulator TNFAIP3 (A20) is inactivated by somatic mutations and genomic deletions in marginal zone lymphomas. *Blood*. 2009;113:4918-4921.
26. Udomsakdi-Auewarakul C, et al. Hepatitis C virus infection in patients with Hodgkin lymphoma in Thailand. *Blood*. 2000;95:3640-3641.
27. Pileri P, et al. Binding of hepatitis C virus to CD81. *Science*. 1998;282:938-941.
28. Cocquerel L, et al. CD81-dependent binding of hepatitis C virus E1E2 heterodimers. *J Virol*. 2003;77:10677-10683.
29. Salido M, et al. Cytogenetic aberrations and their prognostic value in a series of 330 splenic marginal zone B-cell lymphomas: a multicenter study of the Splenic B-Cell Lymphoma Group. *Blood*. 2010;116:1479-1488.
30. Rossi D, et al. The coding genome of splenic marginal zone lymphoma: activation of NOTCH2 and other pathways regulating marginal zone development. *J Exp Med*. 2012;209:1537-1551.
31. Mensah AA, et al. Absence of NOTCH1 gene mutations in MALT lymphomas. *Br J Haematol*. 2012;157:382-384.
32. Watkins AJ, et al. An integrated genomic and expression analysis of 7q deletion in splenic marginal zone lymphoma. *PLoS One*. 2012;7:e44997.
33. Fresquet V, et al. High-throughput sequencing analysis of the chromosome 7q32 deletion reveals IRF5 as a potential tumour suppressor in splenic marginal-zone lymphoma. *Br J Haematol*. 2012;158:712-726.
34. Arribas AJ, et al. Splenic marginal zone lymphoma: comprehensive analysis of gene expression and miRNA profiling. *Mod Pathol*. 2013;26:889-901.
35. Arribas AJ, et al. Nodal marginal zone lymphoma: gene expression and miRNA profiling identify diagnostic markers and potential therapeutic targets. *Blood*. 2012;119:e9-e21.
36. Nakamura S, et al. Long-term clinical outcome of gastric MALT lymphoma after eradication of *Helicobacter pylori*: a multicentre cohort follow-up study of 420 patients in Japan. *Gut*. 2012;61:507-513.
37. Ternynck T, et al. Comparison of normal and CLL lymphocyte surface Ig determinants using peroxidase-labeled antibodies. I. Detection and quantitation of light chain determinants. *Blood*. 1974;43:789-795.
38. Damle RN, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 1999;94:1840-1847.
39. Crespo M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348:1764-1775.
40. Chen L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100:4609-4614.
41. Wiestner A, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood*. 2003;101:4944-4951.
42. Oscier DG, et al. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood*. 2002;100:1177-1184.
43. Migliazza A, et al. Nucleotide sequence, transcription map, and mutation analysis of the 13q14 chromosomal region deleted in B-cell chronic lymphocytic leukemia. *Blood*. 2001;97:2098-2104.
44. Calin GA, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*. 2002;99:15524-15529.
45. Dohner H, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med*. 2000;343:1910-1916.
46. Perez-Galan P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. *Blood*. 2011;117:26-38.
47. Bosch F, et al. PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood*. 1994;84:2726-2732.
48. Marzec M, et al. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. *Blood*. 2006;108:1744-1750.

49. Wiestner A, et al. Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. *Blood*. 2007;109:4599-4606.
50. Chen RW, et al. Truncation in CCND1 mRNA alters miR-16-1 regulation in mantle cell lymphoma. *Blood*. 2008;112:822-829.
51. Alt JR, et al. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev*. 2000;14:3102-3114.
52. Dal Col J, et al. Distinct functional significance of Akt and mTOR constitutive activation in mantle cell lymphoma. *Blood*. 2008;111:5142-5151.
53. Barbash O, et al. Mutations in Fbx4 inhibit dimerization of the SCF(Fbx4) ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell*. 2008;14:68-78.
54. Quelle DE, et al. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev*. 1993;7:1559-1571.
55. Sherr CJ. Cancer cell cycles. *Science*. 1996;274:1672-1677.
56. Hofmann WK, et al. Altered apoptosis pathways in mantle cell lymphoma detected by oligonucleotide microarray. *Blood*. 2001;98:787-794.
57. Chiarle R, et al. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. *Blood*. 2000;95:619-626.
58. Dreyling MH, et al. Alterations of the cyclin D1/p16-pRB pathway in mantle cell lymphoma. *Cancer Res*. 1997;57:4608-4614.
59. Lwin T, et al. Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas. *Blood*. 2007;110:1631-1638.
60. Shakir R, Ngo N, Naresh KN. Correlation of cyclin D1 transcript levels, transcript type and protein expression with proliferation and histology among mantle cell lymphoma. *J Clin Pathol*. 2008;61:920-927.
61. Raty R, et al. Ki-67 expression level, histological subtype, and the International Prognostic Index as outcome predictors in mantle cell lymphoma. *Eur J Haematol*. 2002;69:11-20.
62. Rosenwald A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. *Cancer Cell*. 2003;3:185-197.
63. Determann O, et al. Ki-67 predicts outcome in advanced-stage mantle cell lymphoma patients treated with anti-CD20 immunochemotherapy: results from randomized trials of the European MCL Network and the German Low Grade Lymphoma Study Group. *Blood*. 2008;111:2385-2387.
64. Garcia M, et al. Proliferation predicts failure-free survival in mantle cell lymphoma patients treated with rituximab plus hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with rituximab plus high-dose methotrexate and cytarabine. *Cancer*. 2009;115:1041-1048.
65. Goy A, et al. Potential biomarkers of bortezomib activity in mantle cell lymphoma from the phase 2 PINNACLE trial. *Leuk Lymphoma*. 2010;51:1269-1277.
66. Hashimoto Y, et al. B-cell development in the thymus is limited by inhibitory signals from the thymic microenvironment. *Blood*. 2002;100:3504-3511.
67. Barth TF, et al. Mediastinal (thymic) large B-cell lymphoma: where do we stand? *Lancet Oncol*. 2002;3:229-334.
68. Rosenwald A, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J Exp Med*. 2003;198:851-862.
69. Schmitz R, et al. Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol*. 2009;4:151-174.
70. Green MR, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood*. 2010;116:3268-3277.
71. Melzner I, et al. Biallelic mutation of SOCS-1 impairs JAK2 degradation and sustains phospho-JAK2 action in the MedB-1 mediastinal lymphoma line. *Blood*. 2005;105:2535-2542.
72. Steidl C, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471:377-381.
73. Eberle FC, et al. Gray zone lymphoma: chromosomal aberrations with immunophenotypic and clinical correlations. *Mod Pathol*. 2011;24:1586-1597.
74. Eberle FC, et al. Methylation profiling of mediastinal gray zone lymphoma reveals a distinctive signature with elements shared by classical Hodgkin's lymphoma and primary mediastinal large B-cell lymphoma. *Haematologica*. 2011;96:558-566.
75. Nelson M, et al. Cytogenetic abnormalities and clinical correlations in peripheral T-cell lymphoma. *Br J Haematol*. 2008;141:461-469.
76. Salaverria I, et al. Genomic profiling reveals different genetic aberrations in systemic ALK-positive and ALK-negative anaplastic large cell lymphomas. *Br J Haematol*. 2008;140:516-526.
77. Iqbal J, et al. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood*. 2010;115:1026-1036.
78. Amin HM, Lai R. Pathobiology of ALK+ anaplastic large-cell lymphoma. *Blood*. 2007;110:2259-2267.
79. Stein H, et al. CD30$^+$ anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. *Blood*. 2000;96:3681-3695.
80. Jundt F, et al. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. *Blood*. 2002;99:3398-3403.
81. Moritake H, et al. C-MYC rearrangement may induce an aggressive phenotype in anaplastic lymphoma kinase positive anaplastic large cell lymphoma: identification of a novel fusion gene ALO17/C-MYC. *Am J Hematol*. 2011;86:75-78.
82. Bohling SD, et al. Analysis of gene expression profile of TPM3-ALK positive anaplastic large cell lymphoma reveals overlapping and unique patterns with that of NPM-ALK positive anaplastic large cell lymphoma. *Leuk Res*. 2008;32:383-393.
83. Lamant L, et al. Gene-expression profiling of systemic anaplastic large-cell lymphoma reveals differences based on ALK status and two distinct morphologic ALK+ subtypes. *Blood*. 2007;109:2156-2164.
84. Merkel O, et al. Identification of differential and functionally active miRNAs in both anaplastic lymphoma kinase (ALK)+ and ALK- anaplastic large-cell lymphoma. *Proc Natl Acad Sci U S A*. 2010;107:16228-16233.
85. Thompson MA, et al. Differential gene expression in anaplastic lymphoma kinase-positive and anaplastic lymphoma kinase-negative anaplastic large cell lymphomas. *Hum Pathol*. 2005;36:494-504.
86. Feldman AL, et al. Overexpression of Syk tyrosine kinase in peripheral T-cell lymphomas. *Leukemia*. 2008;22:1139-1143.
87. Piccaluga PP, et al. Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets. *J Clin Invest*. 2007;117:823-834.
88. Streubel B, et al. Novel t(5;9)(q33;q22) fuses ITK to SYK in unspecified peripheral T-cell lymphoma. *Leukemia*. 2006;20:313-318.
89. Pechloff K, et al. The fusion kinase ITK-SYK mimics a T cell receptor signal and drives oncogenesis in conditional mouse models of peripheral T cell lymphoma. *J Exp Med*. 2010;207:1031-1044.
90. Dierks C, et al. The ITK-SYK fusion oncogene induces a T-cell lymphoproliferative disease in mice mimicking human disease. *Cancer Res*. 2010;70:6193-6204.
91. Martinez-Delgado B, et al. Expression profiling of T-cell lymphomas differentiates peripheral and lymphoblastic lymphomas and defines survival related genes. *Clin Cancer Res*. 2004;10:4971-4982.
92. Cuadros M, et al. Identification of a proliferation signature related to survival in nodal peripheral T-cell lymphomas. *J Clin Oncol*. 2007;25:3321-3329.
93. Ballester B, et al. Gene expression profiling identifies molecular subgroups among nodal peripheral T-cell lymphomas. *Oncogene*. 2006;25:1560-1570.
94. Piva R, et al. Gene expression profiling uncovers molecular classifiers for the recognition of anaplastic large-cell lymphoma within peripheral T-cell neoplasms. *J Clin Oncol*. 2010;28:1583-1590.
95. Agnelli L, et al. Identification of a 3-gene model as a powerful diagnostic tool for the recognition of ALK-negative anaplastic large-cell lymphoma. *Blood*. 2012;120:1274-1281.
96. Zettl A, et al. Genomic profiling of peripheral T-cell lymphoma, unspecified, and anaplastic large T-cell lymphoma delineates novel recurrent chromosomal alterations. *Am J Pathol*. 2004;164:1837-1848.
97. Nagel S, et al. Amplification at 7q22 targets cyclin-dependent kinase 6 in T-cell lymphoma. *Leukemia*. 2008;22:387-392.

98. Thorns C, et al. Chromosomal aberrations in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma unspecified: a matrix-based CGH approach. *Genes Chromosomes Cancer*. 2007;46:37-44.
99. Vasmatzis G, et al. Genome-wide analysis reveals recurrent structural abnormalities of TP63 and other p53-related genes in peripheral T-cell lymphomas. *Blood*. 2012;120:2280-2289.
100. de Leval L, Gisselbrecht C, Gaulard P. Advances in the understanding and management of angioimmunoblastic T-cell lymphoma. *Br J Haematol*. 2010;148:673-689.
101. de Leval L, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood*. 2007;109:4952-4963.
102. Rodriguez-Pinilla SM, et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol*. 2008;32:1787-1799.
103. Quivoron C, et al. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell*. 2011;20:25-38.
104. Lemonnier F, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood*. 2012;120:1466-1469.
105. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. *N Engl J Med*. 2012;366:95-96.
106. Cairns RA, et al. IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. *Blood*. 2012;119:1901-1903.

Giovanni Tonon and Kenneth C. Anderson

30

Multiple Myeloma

Multiple myeloma (MM) is a hematological cancer characterized by the accumulation of neoplastic plasma cells in the bone marrow associated with elevated serum and/or urine monoclonal paraprotein levels. During the course of the disease, patients with MM usually suffer debilitating clinical manifestations linked, directly or indirectly, to the accumulation of tumor plasma cells, including lytic bone lesions, anemia, immunodeficiency, and renal function impairment. MM accounts for more than 10% of all hematological malignancies, representing the second most frequent blood cancer in the United States after non-Hodgkin lymphoma.[1-4] MM is almost always preceded by a condition termed *monoclonal gammopathy of undetermined significance* (MGUS),[5,6] defined by the presence in the serum of a monoclonal paraprotein below 30 g/L, an accumulation of less than 10% plasma cells in the bone marrow, in the absence of clinical manifestations. MGUS is present in 1% of adults over age of 25 and evolves toward malignant MM at a rate of 0.5-3% per year.[7,8]

In the past few years, the use of more intense bone marrow transplant treatment regimens and, most importantly, the introduction in the clinic of novel therapeutic compounds such as thalidomide, the immunoregulatory drug lenalidomide (Revlimid), and the proteasome inhibitor bortezomib have considerably improved the outlook for MM patients, leading to a median overall survival above 10 years for more than 30% of patients.[4] As described herein, the ongoing comprehensive analysis of the genetic lesions affecting tumor cells and the growing awareness of the essential role exerted by the surrounding microenvironment in feeding and sustaining tumor growth is providing novel stratification tools and therapeutic opportunities that will likely further improve the patient outcome while reducing drug-related toxicity.

The Role of Tumor Genetics and the Microenvironment in the Pathogenesis of Multiple Myeloma

Overview of the Genetic Lesions Present in Multiple Myeloma

Unlike other hematological cancers such as leukemias and lymphomas that present a relatively unscathed karyotype, the MM genome is thoroughly reshuffled, more similarly in this regard to cancers of epithelial origin. Indeed, a wide array of genetic lesions have been described in MM, which include chromosome gains or losses, Ig-related chromosomal translocations, gains or losses of small chromosomal segments, and finally genetic and epigenetic modifications affecting single genes.

Amid this complexity, however, a classification framework has emerged, with important prognostic and predictive implications[9-14] (Figure 30-1). A first major distinction exists between hyperdiploid (HD) and nonhyperdiploid (NHD) MM. Approximately half of patients present a hyperdiploid karyotype (number of chromosomes ranging from 48 to 74) with concomitant gains of several odd chromosomes such as 3, 5, 7, 9, 11, 15, 19, or 21, in various combinations. The mechanism leading to this peculiar pattern is still unexplained and has no similarities in any other tumor—with the possible exception of a subgroup of acute lymphoblastic leukemia (ALL) patients, who present with concomitant gains of even chromosomes.[15] The remaining patients belong to the NHD group, which includes cases with hypodiploid, pseudodiploid, near-diploid, or tetraploid karyotypes.[13] Importantly, already at the MGUS stage patients present either an HD or an NHD karyotype,[16] which is maintained during the progression of the disease.[17]

Alongside chromosomal gains and losses, MM presents specific chromosomal translocations, so-called primary translocations, as they are again present from the MGUS phase.[12,18] These translocations involve the immunoglobulin H (IgH) locus (at 14q32.3)[19] and less frequently the IgL locus (2p12, kappa or 22q11, lambda)[20] and juxtapose strong Ig enhancers to various genes, resulting in their increased expression.[18] These rearrangements are generally mutually exclusive, although in 5% of MGUS and 25% of advanced MM cases, two independent translocations can be found in the same patient.[9] These primary translocations are for the most part confined to the NHD group.[21] In summary, MM patients are classified into two major groups, HD and NHD, and within the NHD further divided into subgroups based on the presence of specific, primary chromosomal translocations.

Over this general framework, additional genetic or epigenetic modifications are present in the MM genome, at times linked to specific patient subgroups or endowed with

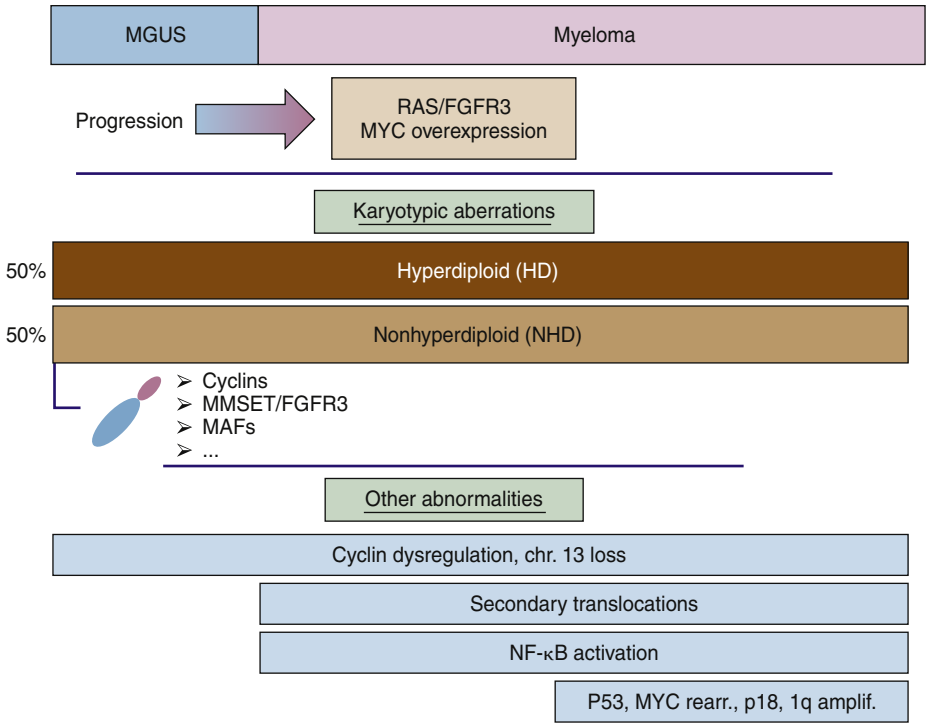


Figure 30-1 An outline of the main factors leading from multiple myeloma (MM) to monoclonal gammopathy of undetermined significance (MGUS); the distinction between HD and NHD MM; and finally additional frequent aberrations reported in MGUS and MM patients.

prognostic relevance. These lesions affect chromosomal regions (e.g., the short and long arm of chromosome 1, chromosome 13, or the short arm of chromosome 17, where the tumor suppressor TP53 is located), or single genes, such as mutations of members of the RAS or NFκB family, or dysregulated expression or chromosomal rearrangements affecting c-MYC. Focal, recurrent copy number aberrations (CNAs) have also been reported, in both cell lines and primary MM tumors, often involving regions harboring established cancer genes.[10, 22-25] For example, deletions affecting well-known or putative tumor suppressor genes such as TP53, UTX, FAM46C, and the NFκB family members BIRC2, BIRC3, and CYLD, or focal amplifications of areas including oncogenes such as MYC, HGF, MCL1, and IL6R, have been reported. Finally, recent next-generation sequencing efforts have identified a wealth of novel somatic mutations involving genes whose role in MM is not yet stringently validated.

Chromosomal Translocations in Multiple Myeloma

Chromosomal translocations in B-cell tumors including MM do not give raise to fusion chimeric proteins, but usually lead to the dysregulated expression of oncogenes through the juxtaposition of their promoters with Ig regulatory elements, via the B-cell–specific mechanisms of switch recombination and somatic hypermutation.[12,18] The primary translocations most frequently identified in MM patients include t(11;14)(q13;q32), which dysregulates the expression of the cyclin D1 gene,[26] with an incidence of approximately 15% in MGUS[27,28] and in MM.[29,30] Another translocation, t(6;14)(p21;q32), induces the dysregulation of cyclin D3 in 2% to 3% of MM patients.[31] t(4;14) is detected in approximately 15% of patients[32] and presents peculiar features. As a result of the translocation, the enhancers Eμ (at the 5′) and Eα (3′) are separated and dysregulate the expression of the juxtaposed genes on both derivative chromosomes, at der(4) and der(14). Hence, on der(14), the 3′ Eα enhancer increases the expression of the receptor tyrosine kinase fibroblast growth factor receptor 3 (FGFR3) gene. On der(4), the 5′ Eμ enhancer drives the expression of the histone methyltransferase Wolf-Hirschhorn syndrome candidate 1 gene (*WHSC1*, also called Multiple Myeloma Set Domain [*MMSET*]). MMSET is almost always upregulated in t(4;14) patients,[33] whereas FGFR3 is not overexpressed in 25% of these patients, suggesting that MMSET represents the main oncogenic culprit of t(4;14) and not FGFR3. Of note, approximately 10% of t(4;14) patients develop activating mutations on FGFR3.[34,35] Finally, the t(14;16)(q32.3;q23) is present in approximately 5% to 10% of MM patients and[12,36] induces overexpression of the oncogene MAF,[37] whereas the (14;20)(q32;q11) has been reported in 2% to 5% of MM cases and affects the family member MAFB.[12,38]

Mutated Genes in Multiple Myeloma

A recent next-generation sequencing (NGS) effort has led to the identification of novel somatic mutations in MM.[39]

This survey has identified on average 35 amino acid–altering point mutations and 21 chromosomal rearrangements per sample, a level of genomic rearrangements again more in line with the degree of mutation rate seen in epithelial cancers such as melanoma and lung carcinomas than in hematological cancers.[40] In addition, as in other blood and epithelial cancers, genes in MM are often mutated at low frequency, suggesting a remarkable and somehow disconcerting intertumor heterogeneity.[40] In fact, in MM, only 10 genes were recurrently mutated.[39] The list included genes where mutations have already been described in MM, such as NRAS (23%), KRAS (26%), and TP53 (8%). Intriguingly mutations were reported also in cyclin D1 (CCND1; 5%), indicating that not only is it frequently translocated and its expression often dysregulated in MM, but it is also mutated (see also later discussion). The remaining six genes include FAM46C (13%), which is also focally deleted; DIS3 (10%); PNRC1 (5%); ALOX12B (8%); HLA-A (5%); and MAGED1 (5%). Mutations affecting these genes have not been previously identified in cancer and reveal novel pathways potentially involved in MM pathogenesis. In particular, two of these genes, DIS3 and FAM46C, are thought to be involved in RNA metabolism and protein homeostasis.

As in other cancers,[41] however, mutations affecting specific genes in MM are in most cases rare; such mutated genes tend to coalesce into specific pathways. In the case of MM, four pathways were significantly enriched in somatic mutations. Confirming previous results,[42,43] genes belonging to the NFκB pathway were frequently mutated. Also, frequent mutations affecting histone-modifying genes such as MLL, MLL2, MLL3, UTX, MMSET, and WHSC1L1 were reported. Mutations affecting the same nucleotide were also found in the IRF4 transcription factor and in its target, PRMD1, confirming previous functional data reporting a prominent role of IRF4 for MM survival.[44] Surprisingly, a significant enrichment in five genes involved in blood coagulation was identified in MM patients. Although a role for this pathway in MM is unknown, thrombin and fibrin have been shown to serve as mitogens in other cell types and have been implicated in metastasis.

Prognostic Implications of Genetic Lesions

Extensive studies in the past 15 years have identified a link between specific genetic lesions and prognosis. HD patients present a better prognosis when compared with NHD MM.[21, 45-47] However, if HD patients acquire chromosome 13 loss and/or gains in the long arm of chromosome 1, patient overall survival worsens considerably.[10, 48, 49] Other acquired genetic lesions in HD have been linked to poor prognosis in this group—for example, IgH translocations, especially those involving unknown partners.[50] Finally, HD patients could evolve toward a pattern characterized by expression of genes associated with high proliferation and poor prognosis.[11]

As for NHD patients, the prognostic outlook varies widely depending on the chromosomal translocation present. Overall, patients with cyclin D translocations tend to present a better prognosis than NHD patients with t(4;14), t(14;16), and t(14;20).

Gains/amplifications of 1q are associated with t(4;14), t(14;16), and possibly chromosome 13 deletion,[10,51] and in general with more proliferative disease states.[52] Several studies have proposed an association between gains/amplification of this region and poor prognosis, based on cytogenetic analysis,[53] fluorescence in situ hybridization (FISH),[38] and expression profiling,[52] as well as aCGH.[10] Importantly, Zhan and colleagues have shown that among a list of 70 genes linked to early disease-related death, there was an enrichment for overexpressed genes mapping to chromosome 1q.[54]

Hemizygous deletions of chromosome 13 are present in MGUS and MM with a similar overall incidence of around 50%.[28,55-58] Given the strong correlation between the presence of 13 loss and other genetic lesions,[30,59] including t(4;14), t(14;16), and NHD, its role as an independent prognostic factor has been questioned.[28,30,46,56,57,60-64]

Finally, aCGH analysis has identified focal regions associated with poor survival containing amplifications on chromosome 8 (involving MYC) and deletion on ch17 (including TP53)— genetic events that have been previously linked to poor prognosis in MM.

It should be emphasized, however, that the significance of any prognostic marker relies heavily on the treatment regimen. In fact, most of the studies just mentioned assessing the prognostic relevance of genetic lesions included patients treated with high-dose chemotherapy followed by bone marrow transplant. A reassessment of the significance of these prognostic and predictive factors is ongoing, in light of the introduction of novel drugs for patient treatment. For example, several of the most well-established genetic prognostic markers failed to show any correlation with prognosis in patients treated with the proteasome inhibitor bortezomib.[65]

The Microenvironment in Multiple Myeloma

Blood cancers develop in secondary lymphoid organs and in the bone marrow (BM). For several acute hematological cancers, such as Burkitt's lymphoma, the genetic lesions underlying these tumor cells are sufficient to promote tumorigenesis; the role of the microenvironment is for the most part marginal. In contrast, mature B-cell malignancies including MM rely heavily on their milieu for their growth and survival[66-68] (Figure 30-2). Indeed, MM cells twist to their advantage

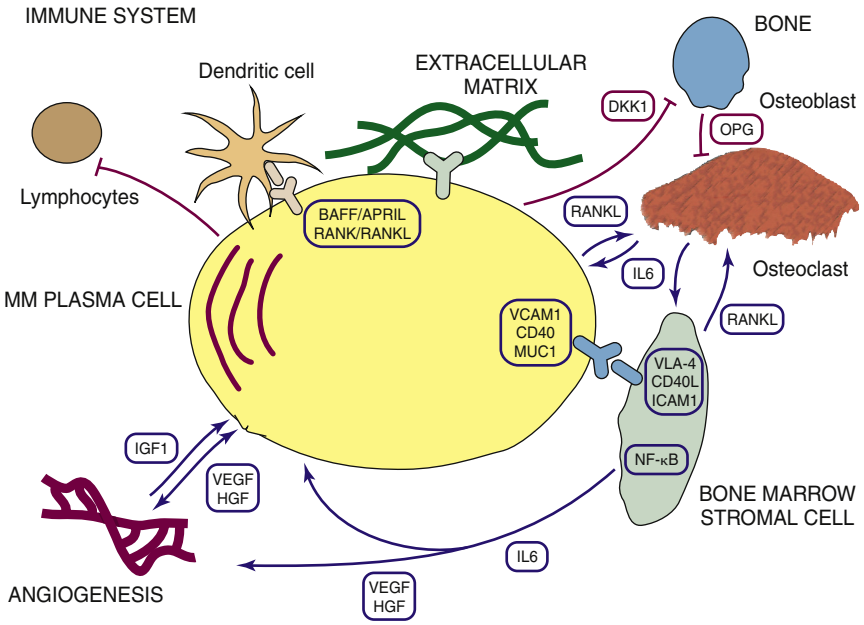


Figure 30-2 A simplified view of the interactions between the multiple myeloma plasma cell and the surrounding environment.

the physiological mechanisms underlying healthy plasma cell homing to the bone marrow and the pathways supporting long-lived plasma cells. MM plasma cells establish tight interactions with essentially all the BM components. The severance of these ties has become an essential therapeutic tool. Indeed, the main mechanism of action of the novel drugs recently introduced into the clinic (thalidomide, lenalidomide, and bortezomib) is their impact on the interactions between MM cells and their cellular counterpart.

The BM microenvironment includes an extracellular matrix (collagen, laminin, fibronectin, and osteopontin) and a rich cellular component. The cellular BM compartment consists of hematopoietic and mesenchymal progenitor and precursor cells, including hematopoietic stem cells (HSCs); BM-derived circulating endothelial precursors (CEPs) and endothelial cells (BMECs); immune cells (dendritic cells, various populations belonging to the B and T lymphocytes, NKT and NK cells, monocytes, and macrophages); erythrocytes; megakaryocytes and platelets; and nonhematopoietic cells, including an ill-defined group of cells labeled fibroblasts/bone marrow stromal cells (BMSCs). Also included are cells involved in bone homeostasis, such as chondroclasts, osteoclasts (OCs), and osteoblasts (OBs).

MM cells interact with BMSC and the extracellular matrix either directly, via adhesion molecules that include LFA1, VLA4, NCAM, ICAM1, and CD44, or indirectly, through chemokines, cytokines, and growth factors released by tumor cells and BMSC, such as interleukin 6 (IL6), insulin-like growth factor 1 (IGF1), tumor necrosis factor-α (TNFα), transforming growth factor-β (TGFβ1), and VEGF. As a result of these multiple-layered interactions, several cancer-relevant pathways become activated in both the tumor and stromal cells, such as NFκB, JAK/STAT, PI3K, and MAPK, establishing powerful positive feedback loops that further increase MM growth and survival. IL6 represents a central cytokine for the growth and survival of MM cells, although IL6-independence could ensue in the late stage of the disease. IL6 engages its receptor (IL6R), leading to the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the proliferation-associated MAPK cascade, and the PI3K/AKT pathway. Moreover, interaction of MM cells with BMSC induces the secretion of IL6 from BMSC through the activation of NFκB. Another paracrine factor that has recently emerged as crucial for MM development is IGF1. Stimulation of MM cell lines with IGF enhances cell proliferation and prevents apoptosis, again through the activation of the MAPK, PI3/AKT, and NFκB pathways. Of note, inhibitors against the receptors of these cytokines and growth factors have been developed, and clinical trials are ongoing to test their effectiveness as novel drugs.

The homing of MM cells to the BM triggers a strong angiogenic response.[69,70] Indeed, it has been shown that angiogenesis correlates with high MM proliferation index, with the more advanced stages of the disease, and ultimately with prognosis. The adhesion of MM cells to the BMECs increases the secretion of several pro-angiogenic cytokines, most importantly of VEGF, basic fibroblast growth factor (bFGF), and matrix metalloproteinases. Conversely, BMECs secrete growth factors, including VEGF, IL6, and IGF1.

Osteolytic bone lesions are a hallmark of MM and are associated with pathologic fractures, bone pain, and diffuse osteoporosis. MM cells increase the number and the activity

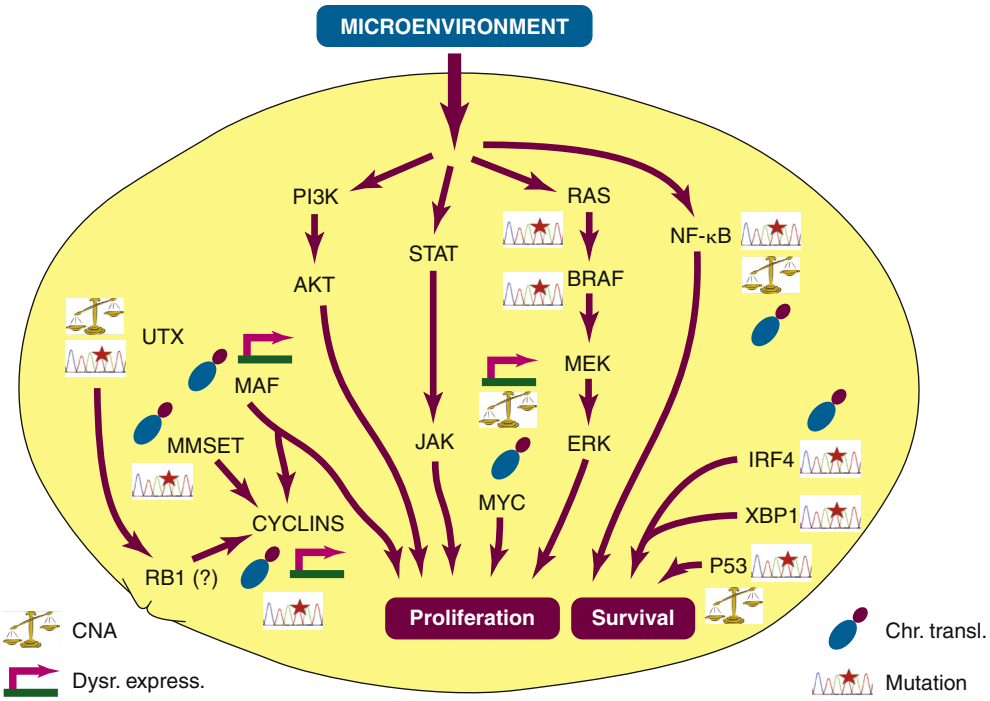


Figure 30-3 Main signaling pathways and genes activated or genetically altered in multiple myeloma. Four different types of aberrations are included: somatic mutations, chromosomal translocations *(Chr. transl)*, copy number aberrations *(CNA)*, and dysregulated expression *(Dysr. express.)*.

of OCs, while reducing the number of OBs, tilting the balance toward increased bone reabsorption. An array of factors that activates the OC is produced by both tumor as well as stromal cells. These factors include macrophage inflammatory protein-1a (MIP-1a) and receptor of NFκB ligand (RANKL). OC activity in turn modulates MM cell growth and survival via the secretion of IL6. Conversely, MM cells impair the maturation of OB cells from mesenchymal stem cells, through the secretion of the WNT-signaling antagonist DKK1, an inhibitor of OB differentiation. In addition, the binding of VLA4 on MM cells to VCAM1 on osteoblast progenitors reduces the levels of the transcription factor RUNX2, essential for OB maturation. Osteoblasts not only preserve the bone structure, but also inhibit MM growth both in vitro and in vivo. Therefore, restoring the number and activity of OBs may increase bone formation as well as provide an antitumoral effect.

Immuno-based tumor surveillance is severely impaired in MM.[71] Both the T and B responses are affected. Specifically, the expansion of regulatory T cells (Treg), reduced T-cell cytotoxic activity and responsiveness to IL2, and defects in B-cell immunity have been reported. These effects are the results of cytokines produced by BMSC, including VEGF, HGF, fibroblast growth factor (FGF), and stromal-cell–derived factor (SDF)-1α. Recently Chauhan and associates have demonstrated a significant enrichment of plasmacytoid dendritic cells (pDCs) in the BM of MM patients. These cells mediate immune deficiency characteristics of MM and are able to promote MM cell growth, survival, and migration and to enhance drug resistance.[72]

Deregulated Pathways in Myeloma and the Opening of Novel Therapeutic Opportunities

The knowledge accumulated in recent years allows a clearer definition of the pathways activated in MM tumor cells, either due to genetic lesions inside the tumor cells or emerging from activating signals from the surrounding microenvironment (Figure 30-3). In several cases, this knowledge has been turned to the patient's advantage, because it has allowed the design of novel targeted therapies specifically addressing single lesions.

MYC

Genetic rearrangements involving the MYC (c-MYC) locus are frequent in the more advanced stages of MM. The anatomy of these lesions has been defined.[73-75] In 25% of cases, the IgH or IgL locus is juxtaposed with the MYC sequence.[76] The pattern, however, is more complex than the classical reciprocal translocations present in the so-called primary translocations.[18,76] Amplifications, inversions and insertions without apparent translocations,[10,23,74,76] or translocations not involving the Ig loci[74,75] have also been described. In a large patient population, rearrangements affecting c-MYC have been reported with a frequency of 3% in MGUS and 10% to 16% in MM.[73,76] MM cell lines have more frequent rearrangements of the c-MYC locus, ranging from 55%[76] to more than 90%, depending on the study.[73,74] Hence, genetic rearrangements affecting the MYC locus likely represent late events in the course of the disease.

Recent lines of evidence suggest that MYC overexpression might represent an early event, at the critical junction

between MGUS and MM. This dysregulated expression seems independent from genetic rearrangements. MYC is overexpressed in a large proportion of MM patients, when compared with plasma cells derived from MGUS patients and healthy individuals, in the absence of evident genetic rearrangements affecting the MYC locus.[10,77,78] Indeed, Chng and colleagues have developed an MYC activation signature and have demonstrated that MYC is activated in up to 67% of MM patients, but not in MGUS. Intriguingly, the MYC activation signature was present in almost all tumors with RAS mutations and was associated with hyperdiploid MM and shorter survival. Importantly, bortezomib treatment was able to overcome the survival disadvantage in patients with MYC activation.[77] In a mouse model of MM, the Vk*MYC model, somatic hypermutation activates an MYC transgene inducing a phenotype closely resembling indolent MM.[78] These data support the notion that dysregulated expression of MYC might be sufficient for MGUS to convert into MM.

MYC is a transcription factor, therefore considered refractory to direct pharmacological inhibition.[79] Recently, Delmore and co-workers have ingeniously devised a method to inhibit MYC target genes.[80] A compound, JQ1, was designed, interfering with the acetyl-lysine recognition domains (bromodomains) of putative coactivator proteins implicated in transcriptional initiation and elongation. Surprisingly, JQ1 downregulated the transcription of the MYC gene itself, followed by genome-wide downregulation of Myc-dependent target genes, ultimately leading to potent antiproliferative effect associated with cell-cycle arrest and cellular senescence.

MAF

Two chromosomal translocations involve the MAF family gene, namely, MAF in the t(14;16)(q32.3;q23),[37] and MAFB in the (14;20)(q32;q11).[12,38] Despite their low incidence, the study of MM patient cells endowed with these translocations has provided important perspectives on the biology of the MM cell and the modality of its interaction with the microenvironment. Both translocations share an overlapping gene expression signature,[11] suggesting a similar repository of downstream targets. The oncogenic role of c-MAF in MM is established. Forced overexpression of MAF enhances myeloma proliferation, probably through the increased expression of cyclin D2, a MAF target gene consistently overexpressed in this group of patients.[9] In contrast, MAF knockdown reduced tumor formation in immune-deficient mice. Another direct MAF target, integrin β7, increases MM cell adhesion to the BM stroma and induces high levels of VEGF. Interestingly, therefore, MAF not only has a direct oncogenic activity within the MM tumor cell, but also affects the microenvironment to the tumor's advantage. An additional report has proposed ARK5, an AMP-activated protein kinase (AMPK)-related protein kinase mediating AKT signals, as a target of MAF and MAFB signaling.[81]

Surprisingly, overexpression of c-MAF has been reported even in the absence of the translocation in up to 30% of patients.[82,83] A recent report has suggested that this upregulation results from the activation of the MEK/ERK/AP-1 axis, downstream of MMSET, in the t(4;14) patient group.[84] Intriguingly, treatment of MM cell lines with a MEK inhibitor selectively induced apoptosis in MAF-expressing MM, providing a molecular rationale for the clinical evaluation of MEK inhibitors in this subgroup of patients.

Previous reports have suggested a reduced incidence of bone disease in this group of patients.[9,11] Two genes might be responsible for this phenotype. The gene DKK1, whose overexpression has been implicated in MM-related bone disease,[85] is significantly downregulated specifically in patients presenting with MAF and MAFB translocations.[11] Moreover, Robbiani and colleagues have reported that the gene osteopontin (OPN) inversely correlates with MM bone disease and is specifically overexpressed in patients with translocations affecting the MAF genes.[86]

Cyclins

Cyclin D dysregulation is a universal phenomenon in MGUS and MM, somehow surprisingly given the exceedingly low proliferation index of both conditions. Among the most recurrent chromosomal rearrangements, two involve cyclins directly: t(11;14)(q13;q32), which occurs in 15% to 20% of MM patients and induces the overexpression of cyclin D1,[26,87-89] and t(6;14)(p21;q32), present in 2% to 3% of MM cases, which increases the expression of cyclin D3.[31] In addition, patients presenting with t(4;14), t(14;16), and t(14;20) demonstrate cyclin D2 overexpression, which in the t(14;16) and t(14;20) patients has been directly linked to MAF/MAFB dysregulation.[9] By unknown mechanisms, HD patients usually show overexpression of cyclin D1, alone or in combination with overexpression of cyclin D2, whereas a subset of HD patients instead produce cyclin D2 alone. It should be noted that the few patients who do not show an evident cyclin dysregulation present RB1 inactivation, supporting the notion that the axis RB1/cyclin is universally derailed in MGUS and MM patients.[8]

Chromatin Remodeling Genes

Chromatin remodeling genes are among the most frequently mutated genes in the cancer genome. Remarkably frequent genetic lesions have been identified in genes affecting DNA methylation (e.g., DNMT3A in AML[90]), nucleosome remodelers (ARID1A in ovarian cancer[91]), and histone-modifying genes in a variety of tumors.[92] In particular, histone posttranslational modifications affecting the N termini of histones 3 and 4, such as acetylation and methylation, are heritable changes

that profoundly affect chromatin structure and gene expression. Genetic lesions involving enzymes that add or remove methylation marks from histone tails have been described, including, as mentioned, MMSET and WHSC1L1, UTX, MLL, MLL2, and MLL3.

MMSET

The recurrent translocations t(4;14)(p16;q32) involve FGFR3[93] and MMSET genes.[94] As mentioned earlier, MMSET seems to represent the real target of this translocation—a notion further confirmed by loss-of-function studies, where downregulation of MMSET with specific shRNA decreased growth and viability of t(4;14) MM cells.[95,96] MMSET presents several alternative spicing variants. Intriguingly, within the gene, the breakpoint position can vary, giving rise to different overexpressed transcripts in t(4;14) samples. MMSET interacts and is likely involved in pathways with a clear relevance in carcinogenesis. MMSET interacts with repressors including SIN3A and histone deacetylases HDAC1 and HDAC2.[95,97,98] However, the oncogenic mechanism underlying MMSET tumorigenic activity remains incompletely understood. Recently it has been shown that t(4;14) MM cases demonstrate an open, permissive chromatin state, associated with high H3K36 di- and trimethylation with concomitant low levels of the trimethylated H3K27.[96] This altered chromatin status was associated with the dysregulated expression of genes involved in cell cycle, apoptosis, DNA repair, and adhesion. Along these lines, t(4;14)-driven MM cases present a remarkable increase in DNA methylation across the genome, a pattern unique in MM.[99]

Finally, a connection between MMSET and the DNA damage response has been recently uncovered.[100] H4K20 methylation increases locally on the induction of double-strand breaks (DSB). Pei and associates have demonstrated that MMSET is responsible for this methylation, in turn facilitating 53BP1 recruitment. MMSET therefore represents a central hub in the DNA damage checkpoint activation and response.

MMSET belongs to a gene family that includes two other genes, WHSC1L1 and NSD1, with ties to cancer. Indeed, somatic mutations in WHSC1L1 have been identified in MM patients as well.[39] This gene is also involved in a chromosomal translocation in acute myeloid leukemia, t(8;11)(p11.2;p15)[101]; is amplified in breast cancer[102] and in lung cancer; and is endowed with oncogenic activity.[103] NSD1 participates in a fusion protein resulting from a chromosomal translocation in acute myeloid leukemia.[104]

UTX

UTX represents the first histone demethylase found mutated or deleted in cancer, including multiple myeloma,[105] renal carcinoma, transitional cell carcinoma of the bladder, chronic myelomonocytic leukemia, acute lymphoblastic leukemia, and prostate cancer. Intriguingly, MM is the tumor where this gene is most frequently inactivated, because truncating mutations or deletions in MM are present in up to 30% of patients[105] (also G. Tonon, unpublished data).

UTX is a JmjC-class enzyme that demethylates di- and trimethylated H3K27me, counteracting the activity of Polycomb complexes (PcGs)[106]—in particular of the histone methyltransferase enhancer of zeste homologue 2 (EZH2), which mediates H3K27 methylation, a transcription-repressive mark. Although limited information is available, one of the main tumorigenic results stemming from UTX inactivation likely results from the unbridled activity of EZH2, leading to enhanced H3K27 trimethylation. EZH2 is highly expressed in several cancer types, including breast, prostate, and lymphomas, and its expression levels correlate with advanced stages of tumor progression and poor prognosis.[107,108] In MM, EZH2 is often overexpressed. It was reported as one of the 30 genes able to distinguish normal plasma cells from MGUS and aggressive myeloma.[109] More recently, activating oncogenic mutations affecting EZH2 were identified in lymphoma.[110,111] In MM, EZH2 is induced by IL6 and enhances proliferation in MM cell lines, whereas its inhibition induces apoptosis.[112]

In addition, UTX has been implicated in cellular differentiation and growth control through transcriptional regulation of the RB1 pathway.[113] Chromatin immunoprecipitation (ChIP)-on-chip experiments have revealed that UTX is present on the promoters of RB1 pathway genes, exerting a transcriptional control on cellular proliferation and mediating cell cycle arrest of primary human fibroblasts,[113] although it remains to be demonstrated whether a similar, RB1-mediated proliferation effect is present also in MM cases with UTX inactivation.

Finally, UTX regulates HOX gene expression, whose orderly activation is essential for normal hematopoiesis.[114] HOXA genes are abnormally expressed in MM.[115] In particular, HOXA9 is normally silenced by trimethylation of H3K27 during hematopoietic differentiation and is consistently upregulated in MM patients. On HOXA9 knockdown, MM cells exhibit a competitive disadvantage.[39] These data suggest that UTX inactivation may dysregulate the expression of HOXA genes, in particular of HOXA9, contributing to the unrestrained proliferation of MM cells.

RB1, P18, and Other Members of the RB1 Pathway

The comprehensive cyclin D dysregulation detected in MM suggest a derailed activity of the RB1 axis. The tumor suppressor RB1 is located on chromosome 13, and a significant fraction of MM patients present with one copy of this chromosome. Nevertheless, a direct role of RB1 inactivation

in MM is uncertain, as inactivating mutations of this gene have not been consistently identified among patients with chromosome 13 hemizygous deletions, or focal homozygous deletions affecting the RB1 region.[12]

Other members of the RB1 pathway are altered in MM, but the pathogenetic and clinical relevance of these aberrations has not been fully elucidated. For example, the tumor suppressor CDKN2A (p16) is usually not deleted in MM,[10,22-24,116] but is methylated in 20% to 30% of MGUS and MM cases.[117-121]

On the other hand, the role of CDKN4C (p18) is more established. Overexpression of p18 in MM cell lines lacking p18 reduced proliferation, whereas it had no effect in a cell line where p18 is normally expressed.[122] p18 is homozygously deleted in up to 38% of MM cell lines and in 2% of MM tumors, but this percentage goes up to 10% in tumors with highest proliferation, as evaluated with an expression profiling signature surrogate.[122,123] Intriguingly, p18 is often overexpressed in the most proliferative MM,[122] and focal deletions at 1p32.3, where p18 resides, are associated with poor prognosis.[124]

NFκB

Lesions involving genes belonging to both the classical and alternative NFκB pathways are frequently detected in MM, namely, in 17% of patients and in approximately 40% of MM cell lines.[39,42,43] The two pathways are tightly interconnected: many signals activate both branches, and many effector proteins or target genes are shared by both cascades. As for the genetic lesions, the most common event consists of inactivating mutations of TRAF3, occurring in 13% of MM patients.[43] Other negative regulators such as TRAF2, BIRC2 and BIRC3, and CYLD carry inactivating mutations or focal homozygous deletions. Chromosome translocations and amplifications involving NFκB-inducing kinase (NIK),[42] CD40, LTBR, TACI, NFKB1, and NFKB2[43] were reported, and all resulted in constitutive activation of either canonical or noncanonical pathways. A more recent study demonstrated that compensatory and/or cooperative effects occur in MM cell lines harboring such alterations.[125] Interestingly, constitutive activation of either canonical or alternative pathways resulted in the regulation of similar sets of genes and biological pathways,[125] conferring increased autonomy from the microenvironment.

The high prevalence of mutations in NFκB family members in MM cell lines compared to MM patients suggests that alterations in the NFκB pathway are relatively late events. Moreover, NFκB dysregulation mainly occurs in NHD compared to HD patients. Of note, part of the activity of the proteasomal inhibitor bortezomib, widely used in MM treatment, is related to the inactivation of the NFκB pathway.[65]

TP53 Deletion and Mutations

Mutations in the TP53 tumor suppressor gene have been reported in MM, with a frequency ranging from 2% to 20%.[39,126-129] Although the TP53 mutation frequency has been reported to be low in MGUS,[129] it increases during the progression of the disease, approaching 80% in MM cell lines,[130] suggesting that TP53 inactivation is a late event in MM. A strong association is present between TP53 mutations and poor prognosis.[131]

Other studies have used deletions in 17p13 (mostly hemizygous) as a surrogate for the inactivation of the TP53 pathway and reported a frequency that in most cases is around 10%,[56,132-134] with a strong association with poor prognosis.[10,56,133-135] Half of MM patients with TP53 mutations had concomitant hemizygous losses at 17p13, whereas only 16% of patients with 17p13 hemizygous deletions had mutations in the remaining copy of TP53.[131] Therefore, it is still unclear whether the TP53 pathway is silenced in MM cases with hemizygous 17p13 deletions when no mutations are detected in the remaining copy of TP53. Moreover, whether deletions on 17p13 are indeed a surrogate for TP53 inactivation or are associated with other, yet undetermined, tumor suppressor pathways is still unclear.

The Proteasome Achilles' Heel

MM cells present a high protein turnover. Protein metabolism in MM cells is finely tuned to prevent overloading that could lead to apoptosis. Compounds targeting different steps of this metabolic process have become the cornerstone of the treatment of this disease, prominent among them the proteasome inhibitor bortezomib, a major breakthrough in the treatment of MM. Among the targets of the proteasome inhibitors are the IL6[136] and NFκB pathways.[137] Although mutations or, more generally, genetic lesions directly affecting the proteasome have not been reported, a recent study has suggested that 1q amplification in MM could be associated with a general dysregulation of genes belonging to the proteasome pathway, in particular of PSMD4, leading to increased resistance to bortezomib.[90] It has been proposed that the recently identified mutations in DIS3, FAM46C, XBP1, and LRRK2 may directly affect the control of protein homeostasis,[39] although this hypothesis needs to be confirmed experimentally.

The Molecular Basis of the Evolution from MGUS to MM

The mechanisms underlying the progression from MGUS to MM are still incompletely understood.[8] From a genetic standpoint, the two conditions are remarkably similar. Both

present either HD or NHD karyotypes,[16] although with modest differences in frequencies with MM; IgH/IgL chromosomal translocations; and deletions of chromosome 13.[27,28,138] Also at the transcriptome level, the two conditions are intriguingly similar, including the pattern of cyclin D expression. Indeed, gene expression profiling has repeatedly failed to discriminate between MGUS and MM.[109,139] Of interest, Zhan and colleagues have been able to show how a subset of MM, featuring a better prognosis, had an expression signature similar to MGUS. On the other hand, a small subset of MGUS clustered together with MM,[140] pointing to a subset of MGUS patients whose disease is potentially more prone to evolve into MM.

Notwithstanding, in the absence of defined phenotypic or genetic differences between MGUS and MM, it has been difficult to stratify MGUS patients, to predict their progression toward MM. Multivariate models have been proposed to address this issue, of crucial relevance from a clinical standpoint.[141,142] In particular, one study has shown that the progression risk ranges from 0.40% to 12%, based on BM plasma–cell flow cytometric immunophenotypic profiles.[141] More extensive studies will be needed to definitively validate these approaches and provide a robust tool that will be invaluable to patients and doctors to predict the progression of MGUS and dictate the treatment to be chosen.

Among the few genetic changes reported in MM and not present (or present with a lower incidence) in MGUS are mutations of two members of RAS family (NRAS and KRAS) at codons 12, 13, and 61 in 40% to 55% of MM, but in only a minority of MGUS patients,[143-148] suggesting a major role for the activation of the MAPK pathway in the progression from MGUS to MM. KRAS has never been reported to be mutated in MGUS, whereas NRAS is mutated in 7% of cases, pointing to a different role of KRAS versus NRAS in MGUS progression.[8]

t(4;14) seems to be more often present in MM than in MGUS.[9, 28, 30, 140, 149, 150] Moreover, the activating mutations affecting FGFR3 are mutually exclusive of the RAS mutations. These data point to MAPK pathway activation as a critical step at the transition from MGUS to MM, mediated by RAS or FGFR3 mutations, or, when neither of these mutations is present, by still unknown mechanisms.

Finally, as mentioned earlier, the dysregulated expression of MYC likely represents an additional universal mechanism driving the MGUS-to-MM progression.

Final Remarks

The general outline of the early events occurring in MM has been greatly elucidated in the past decade. Several questions, however, of critical relevance for the understanding of the pathogenesis of this disease and for the design of novel, more effective treatments, remain to be fully addressed. For example, little is known about the role of the primary events, including cyclin D overexpression and MMSET activity, in the early pathogenesis of the disease. Indeed, the emerging role of epigenetics in MM, and more generally in cancer, warrants extensive studies, given the potential therapeutic implications. Knowledge of the additional lesions promoting the progression of MGUS to MM and, within MM, toward a more aggressive and proliferative disease are still largely incomplete and deserve additional inquiries, given the potential for patient stratification and novel clinical approaches.

The tight interactions between MM cells and BM in the past few years have been extensively studied. Indeed, it could be argued that a considerable part of the success obtained by new compounds introduced in the clinic, such as thalidomide, lenalidomide, and bortezomib, is due to their activity in interrupting the flow of positive signals that the MM cell receives from its surrounding environment. More focused, targeted approaches to further develop compounds tackling these interactions represent a largely untapped treasure chest for novel therapies.

The more extensive application of next-generation sequencing to single patients, at different stages of their disease and of their treatment history, will likely lead to a significant revolution in the MM field—not only in the elucidation of the pathogenetic events responsible for this disease, but also for defining in much finer detail issues such as clonality and drug resistance in individual patients, ultimately driving therapeutic choices and approaches, as recently proposed in pilot studies.[151,152] In fact, even more importantly, the systematic and comprehensive integration of the data emerging from analyses of the genome, transcriptome, methylome, and miRNome should provide a paradigm shift in diagnosis, prognosis, and treatment, initially with available therapies and in the long term with more personalized therapies.

Acknowledgments

Dr. G. Tonon acknowledges the support from the Associazione Italiana per la Ricerca sul Cancro (AIRC), the Association for International Cancer Research (AICR), and the Cariplo Foundation and is a recipient of a Marie Curie International Reintegration Grant. Dr. K. C. Anderson is an American Cancer Society Clinical Research Professor.

Key References

1. Richardson PG, Barlogie B, Berenson J, et al. Clinical factors predictive of outcome with bortezomib in patients with relapsed, refractory multiple myeloma. *Blood*. 2005;106:2977-2981.
2. Richardson PG, Mitsiades CS, Hideshima T, et al. Novel biological therapies for the treatment of multiple myeloma. *Best Pract Res Clin Haematol*. 2005;18:619-634.
3. Barlogie B, Shaughnessy J, Tricot G, et al. Treatment of multiple myeloma. *Blood*. 2004;103:20-32.
4. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med*. 2011;364:1046-1060.
5. Landgren O, Kyle RA, Pfeiffer RM, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood*. 2009;113:5412-5417.
6. Weiss BM, Abadie J, Verma P, et al. A monoclonal gammopathy precedes multiple myeloma in most patients. *Blood*. 2009;113:5418-5422.
7. Kyle RA, Durie BG, Rajkumar SV, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia*. 2010;24:1121-1127.
8. Zingone A, Kuehl WM. Pathogenesis of monoclonal gammopathy of undetermined significance and progression to multiple myeloma. *Semin Hematol*. 2011;48:4-12.
9. Bergsagel PL, Kuehl WM, Zhan F, et al. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*. 2005;106:296-303.
10. Carrasco DR, Tonon G, Huang Y, et al. High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. *Cancer Cell*. 2006;9:313-325.
11. Zhan F, Huang Y, Colla S, et al. The molecular classification of multiple myeloma. *Blood*. 2006;108:2020-2028.
14. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer*. 2012;12:335-348.
15. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004;350:1535-1548.
16. Chng WJ, Van Wier SA, Ahmann GJ, et al. A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. *Blood*. 2005;106:2156-2161.
17. Chng WJ, Winkler JM, Greipp PR, et al. Ploidy status rarely changes in myeloma patients at disease progression. *Leuk Res*. 2006;30:266-271.
20. Debes-Marun CS, Dewald GW, Bryant S, et al. Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. *Leukemia*. 2003;17:427-436.
21. Fonseca R, Debes-Marun CS, Picken EB, et al. The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. *Blood*. 2003;102:2562-2567.
22. Largo C, Saez B, Alvarez S, et al. Multiple myeloma primary cells show a highly rearranged unbalanced genome with amplifications and homozygous deletions irrespective of the presence of immunoglobulin-related chromosome translocations. *Haematologica*. 2007;92:795-802.
23. Largo C, Alvarez S, Saez B, et al. Identification of overexpressed genes in frequently gained/amplified chromosome regions in multiple myeloma. *Haematologica*. 2006;91:184-191.
24. Walker BA, Leone PE, Jenner MW, et al. Integration of global SNP-based mapping and expression arrays reveals key regions, mechanisms, and genes important in the pathogenesis of multiple myeloma. *Blood*. 2006;108:1733-1743.
25. Dickens NJ, Walker BA, Leone PE, et al. Homozygous deletion mapping in myeloma samples identifies genes and an expression signature relevant to pathogenesis and outcome. *Clin Cancer Res*. 2010;16:1856-1864.
32. Keats JJ, Reiman T, Belch AR, et al. Ten years and counting: so what do we know about t(4;14)(p16;q32) multiple myeloma? *Leuk Lymphoma*. 2006;47:2289-2300.
33. Keats JJ, Maxwell CA, Taylor BJ, et al. Overexpression of transcripts originating from the MMSET locus characterizes all t(4;14)(p16;q32)-positive multiple myeloma patients. *Blood*. 2005;105:4060-4069.
39. Chapman MA, Lawrence MS, Keats JJ, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011;471:467-472.
40. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011;331:1553-1558.
41. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806.
42. Annunziata CM, Davis RE, Demchenko Y, et al. Frequent engagement of the classical and alternative NFkappaB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell*. 2007;12:115-130.
43. Keats JJ, Fonseca R, Chesi M, et al. Promiscuous mutations activate the noncanonical NFkappaB pathway in multiple myeloma. *Cancer Cell*. 2007;12:131-144.
44. Shaffer AL, Emre NC, Lamy L, et al. IRF4 addiction in multiple myeloma. *Nature*. 2008;454:226-231.
48. Agnelli L, Fabris S, Bicciato S, et al. Upregulation of translational machinery and distinct genetic subgroups characterise hyperdiploidy in multiple myeloma. *Br J Haematol*. 2007;136:565-573.
49. Chng WJ, Kumar S, Vanwier S, et al. Molecular dissection of hyperdiploid multiple myeloma by gene expression profiling. *Cancer Res*. 2007;67:2982-2989.
50. Chng WJ, Santana-Davila R, Van Wier SA, et al. Prognostic factors for hyperdiploid-myeloma: effects of chromosome 13 deletions and IgH translocations. *Leukemia*. 2006;20:807-813.
51. Fonseca R, Van Wier SA, Chng WJ, et al. Prognostic value of chromosome 1q21 gain by fluorescent in situ hybridization and increase CKS1B expression in myeloma. *Leukemia*. 2006;20:2034-2040.
52. Chang H, Yeung J, Xu W, et al. Significant increase of CKS1B amplification from monoclonal gammopathy of undetermined significance to multiple myeloma and plasma cell leukaemia as demonstrated by interphase fluorescence in situ hybridisation. *Br J Haematol*. 2006;134:613-615.
53. Wu KL, Beverloo B, Lokhorst HM, et al. Abnormalities of chromosome 1p/q are highly associated with chromosome 13/13q deletions and are an adverse prognostic factor for the outcome of high-dose chemotherapy in patients with multiple myeloma. *Br J Haematol*. 2007;136:615-623.
54. Shaughnessy Jr JD, Zhan F, Burington BE, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. *Blood*. 2007;109:2276-2284.
56. Fonseca R, Blood E, Rue M, et al. Clinical and biologic implications of recurrent genomic aberrations in myeloma. *Blood*. 2003;101:4569-4575.
64. Shaughnessy J, Jacobson J, Sawyer J, et al. Continuous absence of metaphase-defined cytogenetic abnormalities, especially of chromosome 13 and hypodiploidy, ensures long-term survival in multiple myeloma treated with Total Therapy I: interpretation in the context of global gene expression. *Blood*. 2003;101:3849-3856.
65. Mulligan G, Mitsiades C, Bryant B, et al. Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. *Blood*. 2007;109:3177-3188.
66. Burger JA, Ghia P, Rosenwald A, et al. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood*. 2009;114:3367-3375.
67. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia*. 2009;23:10-24.
68. Hideshima T, Mitsiades C, Tonon G, et al. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer*. 2007;7:585-598.
70. Vacca A, Ribatti D. Bone marrow angiogenesis in multiple myeloma. *Leukemia*. 2006;20:193-199.
71. Rutella S, Locatelli F. Targeting multiple-myeloma-induced immune dysfunction to improve immunotherapy outcomes. *Clin Dev Immunol*. 2012;2012:196063.
72. Chauhan D, Singh AV, Brahmandam M, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell*. 2009;16:309-323.

73. Fabris S, Storlazzi CT, Baldini L, et al. Heterogeneous pattern of chromosomal breakpoints involving the MYC locus in multiple myeloma. *Genes Chromosomes Cancer*. 2003;37:261-269.
77. Chng WJ, Huang GF, Chung TH, et al. Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. *Leukemia*. 2011;25:1026-1035.
78. Chesi M, Robbiani DF, Sebag M, et al. AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer Cell*. 2008;13:167-180.
80. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell*. 2011;146:904-917.
81. Suzuki A, Iida S, Kato-Uranishi M, et al. ARK5 is transcriptionally regulated by the Large-MAF family and mediates IGF-1-induced cell invasion in multiple myeloma: ARK5 as a new molecular determinant of malignant multiple myeloma. *Oncogene*. 2005;24:6936-6944.
82. Hurt EM, Wiestner A, Rosenwald A, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell*. 2004;5:191-199.
83. Lombardi L, Poretti G, Mattioli M, et al. Molecular characterization of human multiple myeloma cell lines by integrative genomics: insights into the biology of the disease. *Genes Chromosomes Cancer*. 2007;46:226-238.
84. Annunziata CM, Hernandez L, Davis RE, et al. A mechanistic rationale for MEK inhibitor therapy in myeloma based on blockade of MAF oncogene expression. *Blood*. 2011;117:2396-2404.
85. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med*. 2003;349:2483-2494.
86. Robbiani DF, Colon K, Ely S, et al. Osteopontin dysregulation and lytic bone lesions in multiple myeloma. *Hematol Oncol*. 2007;25:16-20.
90. Shaughnessy Jr JD, Qu P, Usmani S, et al. Pharmacogenomics of bortezomib test-dosing identifies hyperexpression of proteasome genes, especially PSMD4, as novel high-risk feature in myeloma treated with Total Therapy 3. *Blood*. 2011;118:3512-3524.
91. Jones S, Wang TL, Shih Ie M, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330:228-231.
92. Chi P, Allis CD, Wang GG. Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer*. 2010;10:457-469.
95. Marango J, Shimoyama M, Nishio H, et al. The MMSET protein is a histone methyltransferase with characteristics of a transcriptional corepressor. *Blood*. 2008;111:3145-3154.
96. Martinez-Garcia E, Popovic R, Min DJ, et al. The MMSET histone methyl transferase switches global histone methylation and alters gene expression in t(4;14) multiple myeloma cells. *Blood*. 2011;117:211-220.
97. Todoerti K, Ronchetti D, Agnelli L, et al. Transcription repression activity is associated with the type I isoform of the MMSET gene involved in t(4;14) in multiple myeloma. *Br J Haematol*. 2005;131:214-218.
98. Kim JY, Kee HJ, Choe NW, et al. Multiple-myeloma-related WHSC1/MMSET isoform RE-IIBP is a histone methyltransferase with transcriptional repression activity. *Mol Cell Biol*. 2008;28:2023-2034.
99. Walker BA, Wardell CP, Chiecchio L, et al. Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*. 2011;117:553-562.
100. Pei H, Zhang L, Luo K, et al. MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature*. 2011;470:124-128.
103. Tonon G, Wong KK, Maulik G, et al. High-resolution genomic profiles of human lung cancer. *Proc Natl Acad Sci U S A*. 2005;102:9625-9630.
105. van Haaften G, Dalgliesh GL, Davies H, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nat Genet*. 2009;41:521-523.
106. Smith ER, Lee MG, Winter B, et al. Drosophila UTX is a histone H3 Lys27 demethylase that colocalizes with the elongating form of RNA polymerase II. *Mol Cell Biol*. 2008;28:1041-1046.
107. Chang CJ, Yang JY, Xia W, et al. EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. *Cancer Cell*. 2011;19:86-100.
108. Varambally S, Cao Q, Mani RS, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science*. 2008;322:1695-1699.
110. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci U S A*. 2010;107:20980-20985.
111. Yap DB, Chu J, Berg T, et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. *Blood*. 2011;117:2451-2459.
112. Croonquist PA, Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. *Oncogene*. 2005;24:6269-6280.
113. Wang JK, Tsai MC, Poulin G, et al. The histone demethylase UTX enables RB-dependent cell fate control. *Genes Dev*. 2010;24:327-332.
114. Agger K, Cloos PA, Christensen J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature*. 2007;449:731-734.
115. Hudlebusch HR, Lodahl M, Johnsen HE, et al. Expression of HOXA genes in patients with multiple myeloma. *Leuk Lymphoma*. 2004;45:1215-1217.
116. Elnenaei MO, Gruszka-Westwood AM, A'Hern R, et al. Gene abnormalities in multiple myeloma: the relevance of TP53, MDM2, and CDKN2A. *Haematologica*. 2003;88:529-537.
118. Gonzalez-Paz N, Chng WJ, McClure RF, et al. Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. *Blood*. 2007;109:1228-1232.
119. Dib A, Barlogie B, Shaughnessy Jr JD, et al. Methylation and expression of the p16INK4A tumor suppressor gene in multiple myeloma. *Blood*. 2007;109:1337-1338.
120. Ribas C, Colleoni GW, Felix RS, et al. p16 gene methylation lacks correlation with angiogenesis and prognosis in multiple myeloma. *Cancer Lett*. 2005;222:247-254.
121. Kramer A, Schultheis B, Bergmann J, et al. Alterations of the cyclin D1/pRb/p16(INK4A) pathway in multiple myeloma. *Leukemia*. 2002;16:1844-1851.
122. Dib A, Peterson TR, Raducha-Grace L, et al. Paradoxical expression of INK4c in proliferative multiple myeloma tumors: bi-allelic deletion vs increased expression. *Cell Div*. 2006;1:23.
124. Boyd KD, Ross FM, Walker BA, et al. Mapping of chromosome 1p deletions in myeloma identifies FAM46C at 1p12 and CDKN2C at 1p32.3 as being genes in regions associated with adverse survival. *Clin Cancer Res*. 2011;17:7776-7784.
125. Demchenko YN, Glebov OK, Zingone A, et al. Classical and/or alternative NFkappaB pathway activation in multiple myeloma. *Blood*. 2010;115:3541-3552.
131. Chng WJ, Price-Troska T, Gonzalez-Paz N, et al. Clinical significance of TP53 mutation in myeloma. *Leukemia*. 2007;21:582-584.
133. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. *Blood*. 2007;109:3489-3495.
134. Chang H, Qi C, Yi QL, et al. p53 gene deletion detected by fluorescence in situ hybridization is an adverse prognostic factor for patients with multiple myeloma following autologous stem cell transplantation. *Blood*. 2005;105:358-360.
135. Gertz MA, Lacy MQ, Dispenzieri A, et al. Clinical implications of t(11;14)(q13;q32), t(4;14)(p16.3;q32), and -17p13 in myeloma patients treated with high-dose therapy. *Blood*. 2005;106:2837-2840.
136. Davies FE, Dring AM, Li C, et al. Insights into the multistep transformation of MGUS to myeloma using microarray expression analysis. *Blood*. 2003;102:4504-4511.
138. Kaufmann H, Ackermann J, Baldia C, et al. Both IGH translocations and chromosome 13q deletions are early events in monoclonal gammopathy of undetermined significance and do not evolve during transition to multiple myeloma. *Leukemia*. 2004;18:1879-1882.
139. Hardin J, Waddell M, Page CD, et al. Evaluation of multiple models to distinguish closely related forms of disease using DNA microarray data: an application to multiple myeloma. *Stat Appl Genet Mol Biol*. 2004;3(10).

140. Zhan F, Barlogie B, Arzoumanian V, et al. Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. *Blood*. 2007;109:1692-1700.
141. Perez-Persona E, Vidriales MB, Mateo G, et al. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. *Blood*. 2007;110:2586-2592.
142. Rajkumar SV, Kyle RA, Therneau TM, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood*. 2005;106:812-817.
148. Rasmussen T, Kuehl M, Lodahl M, et al. Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors. *Blood*. 2005;105:317-323.
149. Keats JJ, Reiman T, Maxwell CA, et al. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. *Blood*. 2003;101:1520-1529.
151. Egan JB, Shi CX, Tembe W, et al. Whole-genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution, and clonal tides. *Blood*. 2012;120:1060-1066.
152. Keats JJ, Chesi M, Egan JB, et al. Clonal competition with alternating dominance in multiple myeloma. *Blood*. 2012;120:1067-1076.

References

1. Richardson PG, Barlogie B, Berenson J, et al. Clinical factors predictive of outcome with bortezomib in patients with relapsed, refractory multiple myeloma. *Blood*. 2005;106:2977-2981.
2. Richardson PG, Mitsiades CS, Hideshima T, et al. Novel biological therapies for the treatment of multiple myeloma. *Best Pract Res Clin Haematol*. 2005;18:619-634.
3. Barlogie B, Shaughnessy J, Tricot G, et al. Treatment of multiple myeloma. *Blood*. 2004;103:20-32.
4. Palumbo A, Anderson K. Multiple myeloma. *N Engl J Med*. 2011;364:1046-1060.
5. Landgren O, Kyle RA, Pfeiffer RM, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood*. 2009;113:5412-5417.
6. Weiss BM, Abadie J, Verma P, et al. A monoclonal gammopathy precedes multiple myeloma in most patients. *Blood*. 2009;113:5418-5422.
7. Kyle RA, Durie BG, Rajkumar SV, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia*. 2010;24:1121-1127.
8. Zingone A, Kuehl WM. Pathogenesis of monoclonal gammopathy of undetermined significance and progression to multiple myeloma. *Semin Hematol*. 2011;48:4-12.
9. Bergsagel PL, Kuehl WM, Zhan F, et al. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*. 2005;106:296-303.
10. Carrasco DR, Tonon G, Huang Y, et al. High-resolution genomic profiles define distinct clinico-pathogenetic subgroups of multiple myeloma patients. *Cancer Cell*. 2006;9:313-325.
11. Zhan F, Huang Y, Colla S, et al. The molecular classification of multiple myeloma. *Blood*. 2006;108:2020-2028.
12. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. *Nat Rev Cancer*. 2002;2:175-187.
13. Smadja NV, Fruchart C, Isnard F, et al. Chromosomal analysis in multiple myeloma: cytogenetic evidence of two different diseases. *Leukemia*. 1998;12:960-969.
14. Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer*. 2012;12:335-348.
15. Pui CH, Relling MV, Downing JR. Acute lymphoblastic leukemia. *N Engl J Med*. 2004;350:1535-1548.
16. Chng WJ, Van Wier SA, Ahmann GJ, et al. A validated FISH trisomy index demonstrates the hyperdiploid and nonhyperdiploid dichotomy in MGUS. *Blood*. 2005;106:2156-2161.
17. Chng WJ, Winkler JM, Greipp PR, et al. Ploidy status rarely changes in myeloma patients at disease progression. *Leuk Res*. 2006;30:266-271.
18. Bergsagel PL, Kuehl WM. Chromosome translocations in multiple myeloma. *Oncogene*. 2001;20:5611-5622.
19. Bergsagel PL, Chesi M, Nardini E, et al. Promiscuous translocations into immunoglobulin heavy chain switch regions in multiple myeloma. *Proc Natl Acad Sci U S A*. 1996;93:13931-13936.
20. Debes-Marun CS, Dewald GW, Bryant S, et al. Chromosome abnormalities clustering and its implications for pathogenesis and prognosis in myeloma. *Leukemia*. 2003;17:427-436.
21. Fonseca R, Debes-Marun CS, Picken EB, et al. The recurrent IgH translocations are highly associated with nonhyperdiploid variant multiple myeloma. *Blood*. 2003;102:2562-2567.
22. Largo C, Saez B, Alvarez S, et al. Multiple myeloma primary cells show a highly rearranged unbalanced genome with amplifications and homozygous deletions irrespective of the presence of immunoglobulin-related chromosome translocations. *Haematologica*. 2007;92:795-802.
23. Largo C, Alvarez S, Saez B, et al. Identification of overexpressed genes in frequently gained/amplified chromosome regions in multiple myeloma. *Haematologica*. 2006;91:184-191.
24. Walker BA, Leone PE, Jenner MW, et al. Integration of global SNP-based mapping and expression arrays reveals key regions, mechanisms, and genes important in the pathogenesis of multiple myeloma. *Blood*. 2006;108:1733-1743.
25. Dickens NJ, Walker BA, Leone PE, et al. Homozygous deletion mapping in myeloma samples identifies genes and an expression signature relevant to pathogenesis and outcome. *Clin Cancer Res*. 2010;16:1856-1864.
26. Seto M, Yamamoto K, Iida S, et al. Gene rearrangement and overexpression of PRAD1 in lymphoid malignancy with t(11;14)(q13;q32) translocation. *Oncogene*. 1992;7:1401-1406.
27. Fonseca R, Bailey RJ, Ahmann GJ, et al. Genomic abnormalities in monoclonal gammopathy of undetermined significance. *Blood*. 2002;100:1417-1424.
28. Avet-Loiseau H, Facon T, Daviet A, et al. 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myelome. *Cancer Res*. 1999;59:4546-4550.
29. Fonseca R, Blood EA, Oken MM, et al. Myeloma and the t(11;14)(q13;q32): evidence for a biologically defined unique subset of patients. *Blood*. 2002;99:3735-3741.
30. Avet-Loiseau H, Facon T, Grosbois B, et al. Oncogenesis of multiple myeloma: 14q32 and 13q chromosomal abnormalities are not randomly distributed, but correlate with natural history, immunological features, and clinical presentation. *Blood*. 2002;99:2185-2191.
31. Shaughnessy Jr J, Gabrea A, Qi Y, et al. Cyclin D3 at 6p21 is dysregulated by recurrent chromosomal translocations to immunoglobulin loci in multiple myeloma. *Blood*. 2001;98:217-223.
32. Keats JJ, Reiman T, Belch AR, et al. Ten years and counting: so what do we know about t(4;14)(p16;q32) multiple myeloma? *Leuk Lymphoma*. 2006;47:2289-2300.
33. Keats JJ, Maxwell CA, Taylor BJ, et al. Overexpression of transcripts originating from the MMSET locus characterizes all t(4;14)(p16;q32)-positive multiple myeloma patients. *Blood*. 2005;105:4060-4069.
34. Chesi M, Brents LA, Ely SA, et al. Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. *Blood*. 2001;97:729-736.
35. Intini D, Baldini L, Fabris S, et al. Analysis of FGFR3 gene mutations in multiple myeloma patients with t(4;14). *Br J Haematol*. 2001;114:362-364.
36. Rasmussen T, Knudsen LM, Dahl IM, et al. C-MAF oncogene dysregulation in multiple myeloma: frequency and biological relevance. *Leuk Lymphoma*. 2003;44:1761-1766.
37. Chesi M, Bergsagel PL, Shonukan OO, et al. Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma. *Blood*. 1998;91:4457-4463.
38. Hanamura I, Iida S, Akano Y, et al. Ectopic expression of MAFB gene in human myeloma cells carrying (14;20)(q32;q11) chromosomal translocations. *Jpn J Cancer Res*. 2001;92:638-644.
39. Chapman MA, Lawrence MS, Keats JJ, et al. Initial genome sequencing and analysis of multiple myeloma. *Nature*. 2011;471:467-472.
40. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011;331:1553-1558.
41. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806.
42. Annunziata CM, Davis RE, Demchenko Y, et al. Frequent engagement of the classical and alternative NFkappaB pathways by diverse genetic abnormalities in multiple myeloma. *Cancer Cell*. 2007;12:115-130.
43. Keats JJ, Fonseca R, Chesi M, et al. Promiscuous mutations activate the noncanonical NFkappaB pathway in multiple myeloma. *Cancer Cell*. 2007;12:131-144.
44. Shaffer AL, Emre NC, Lamy L, et al. IRF4 addiction in multiple myeloma. *Nature*. 2008;454:226-231.
45. Calasanz MJ, Cigudosa JC, Odero MD, et al. Hypodiploidy and 22q11 rearrangements at diagnosis are associated with poor prognosis in patients with multiple myeloma. *Br J Haematol*. 1997;98:418-425.
46. Fassas AB, Spencer T, Sawyer J, et al. Both hypodiploidy and deletion of chromosome 13 independently confer poor prognosis in multiple myeloma. *Br J Haematol*. 2002;118:1041-1047.
47. Smadja NV, Bastard C, Brigaudeau C, et al. Hypodiploidy is a major prognostic factor in multiple myeloma. *Blood*. 2001;98:2229-2238.

48. Agnelli L, Fabris S, Bicciato S, et al. Upregulation of translational machinery and distinct genetic subgroups characterise hyperdiploidy in multiple myeloma. *Br J Haematol*. 2007;136:565-573.
49. Chng WJ, Kumar S, Vanwier S, et al. Molecular dissection of hyperdiploid multiple myeloma by gene expression profiling. *Cancer Res*. 2007;67:2982-2989.
50. Chng WJ, Santana-Davila R, Van Wier SA, et al. Prognostic factors for hyperdiploid-myeloma: effects of chromosome 13 deletions and IgH translocations. *Leukemia*. 2006;20:807-813.
51. Fonseca R, Van Wier SA, Chng WJ, et al. Prognostic value of chromosome 1q21 gain by fluorescent in situ hybridization and increase CKS1B expression in myeloma. *Leukemia*. 2006;20:2034-2040.
52. Chang H, Yeung J, Xu W, et al. Significant increase of CKS1B amplification from monoclonal gammopathy of undetermined significance to multiple myeloma and plasma cell leukaemia as demonstrated by interphase fluorescence in situ hybridisation. *Br J Haematol*. 2006;134:613-615.
53. Wu KL, Beverloo B, Lokhorst HM, et al. Abnormalities of chromosome 1p/q are highly associated with chromosome 13/13q deletions and are an adverse prognostic factor for the outcome of high-dose chemotherapy in patients with multiple myeloma. *Br J Haematol*. 2007;136:615-623.
54. Shaughnessy Jr JD, Zhan F, Burington BE, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. *Blood*. 2007;109:2276-2284.
55. Shaughnessy J, Tian E, Sawyer J, et al. High incidence of chromosome 13 deletion in multiple myeloma detected by multiprobe interphase FISH. *Blood*. 2000;96:1505-1511.
56. Fonseca R, Blood E, Rue M, et al. Clinical and biologic implications of recurrent genomic aberrations in myeloma. *Blood*. 2003;101:4569-4575.
57. Zojer N, Konigsberg R, Ackermann J, et al. Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. *Blood*. 2000;95:1925-1930.
58. Konigsberg R, Ackermann J, Kaufmann H, et al. Deletions of chromosome 13q in monoclonal gammopathy of undetermined significance. *Leukemia*. 2000;14:1975-1979.
59. Fonseca R, Oken MM, Greipp PR. The t(4;14)(p16.3;q32) is strongly associated with chromosome 13 abnormalities in both multiple myeloma and monoclonal gammopathy of undetermined significance. *Blood*. 2001;98:1271-1272.
60. Desikan R, Barlogie B, Sawyer J, et al. Results of high-dose therapy for 1000 patients with multiple myeloma: durable complete remissions and superior survival in the absence of chromosome 13 abnormalities. *Blood*. 2000;95:4008-4010.
61. Tricot G, Barlogie B, Jagannath S, et al. Poor prognosis in multiple myeloma is associated only with partial or complete deletions of chromosome 13 or abnormalities involving 11q and not with other karyotype abnormalities. *Blood*. 1995;86:4250-4256.
62. Facon T, Avet-Loiseau H, Guillerm G, et al. Chromosome 13 abnormalities identified by FISH analysis and serum beta2-microglobulin produce a powerful myeloma staging system for patients receiving high-dose therapy. *Blood*. 2001;97:1566-1571.
63. Fonseca R, Harrington D, Oken MM, et al. Biological and prognostic significance of interphase fluorescence in situ hybridization detection of chromosome 13 abnormalities (delta13) in multiple myeloma: an Eastern Cooperative Oncology Group study. *Cancer Res*. 2002;62:715-720.
64. Shaughnessy J, Jacobson J, Sawyer J, et al. Continuous absence of metaphase-defined cytogenetic abnormalities, especially of chromosome 13 and hypodiploidy, ensures long-term survival in multiple myeloma treated with Total Therapy I: interpretation in the context of global gene expression. *Blood*. 2003;101:3849-3856.
65. Mulligan G, Mitsiades C, Bryant B, et al. Gene expression profiling and correlation with outcome in clinical trials of the proteasome inhibitor bortezomib. *Blood*. 2007;109:3177-3188.
66. Burger JA, Ghia P, Rosenwald A, et al. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood*. 2009;114:3367-3375.
67. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia*. 2009;23:10-24.
68. Hideshima T, Mitsiades C, Tonon G, et al. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer*. 2007;7:585-598.
69. Vacca A, Ribatti D, Presta M, et al. Bone marrow neovascularization, plasma cell angiogenic potential, and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma. *Blood*. 1999;93:3064-3073.
70. Vacca A, Ribatti D. Bone marrow angiogenesis in multiple myeloma. *Leukemia*. 2006;20:193-199.
71. Rutella S, Locatelli F. Targeting multiple-myeloma-induced immune dysfunction to improve immunotherapy outcomes. *Clin Dev Immunol*. 2012;2012:196063.
72. Chauhan D, Singh AV, Brahmandam M, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell*. 2009;16:309-323.
73. Fabris S, Storlazzi CT, Baldini L, et al. Heterogeneous pattern of chromosomal breakpoints involving the MYC locus in multiple myeloma. *Genes Chromosomes Cancer*. 2003;37:261-269.
74. Shou Y, Martelli ML, Gabrea A, et al. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. *Proc Natl Acad Sci U S A*. 2000;97:228-233.
75. Hollis GF, Gazdar AF, Bertness V, Kirsch IR. Complex translocation disrupts c-myc regulation in a human plasma cell myeloma. *Mol Cell Biol*. 1988;8:124-129.
76. Avet-Loiseau H, Gerson F, Magrangeas F, et al. Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. *Blood*. 2001;98:3082-3086.
77. Chng WJ, Huang GF, Chung TH, et al. Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. *Leukemia*. 2011;25:1026-1035.
78. Chesi M, Robbiani DF, Sebag M, et al. AID-dependent activation of a MYC transgene induces multiple myeloma in a conditional mouse model of post-germinal center malignancies. *Cancer Cell*. 2008;13:167-180.
79. Darnell Jr JE. Transcription factors as targets for cancer therapy. *Nat Rev Cancer*. 2002;2:740-749.
80. Delmore JE, Issa GC, Lemieux ME, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell*. 2011;146:904-917.
81. Suzuki A, Iida S, Kato-Uranishi M, et al. ARK5 is transcriptionally regulated by the Large-MAF family and mediates IGF-1-induced cell invasion in multiple myeloma: ARK5 as a new molecular determinant of malignant multiple myeloma. *Oncogene*. 2005;24:6936-6944.
82. Hurt EM, Wiestner A, Rosenwald A, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer Cell*. 2004;5:191-199.
83. Lombardi L, Poretti G, Mattioli M, et al. Molecular characterization of human multiple myeloma cell lines by integrative genomics: insights into the biology of the disease. *Genes Chromosomes Cancer*. 2007;46:226-238.
84. Annunziata CM, Hernandez L, Davis RE, et al. A mechanistic rationale for MEK inhibitor therapy in myeloma based on blockade of MAF oncogene expression. *Blood*. 2011;117:2396-2404.
85. Tian E, Zhan F, Walker R, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med*. 2003;349:2483-2494.
86. Robbiani DF, Colon K, Ely S, et al. Osteopontin dysregulation and lytic bone lesions in multiple myeloma. *Hematol Oncol*. 2007;25:16-20.
87. Chesi M, Bergsagel PL, Brents LA, et al. Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines. *Blood*. 1996;88:674-681.
88. Akiyama N, Tsuruta H, Sasaki H, et al. Messenger RNA levels of five genes located at chromosome 11q13 in B-cell tumors with chromosome translocation t(11;14)(q13;q32). *Cancer Res*. 1994;54:377-379.
89. Kobayashi H, Saito H, Kitano K, et al. Overexpression of the PRAD1 oncogene in a patient with multiple myeloma and t(11;14)(q13;q32). *Acta Haematol*. 1995;94:199-203.
90. Shaughnessy Jr JD, Qu P, Usmani S, et al. Pharmacogenomics of bortezomib test-dosing identifies hyperexpression of proteasome genes, especially PSMD4, as novel high-risk feature in myeloma treated with Total Therapy 3. *Blood*. 2011;118:3512-3524.

91. Jones S, Wang TL, Shih Ie M, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330:228-231.
92. Chi P, Allis CD, Wang GG. Covalent histone modifications—miswritten, misinterpreted and mis-erased in human cancers. *Nat Rev Cancer*. 2010;10:457-469.
93. Chesi M, Nardini E, Brents LA, et al. Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. *Nat Genet*. 1997;16:260-264.
94. Chesi M, Nardini E, Lim RS, et al. The t(4;14) translocation in myeloma dysregulates both FGFR3 and a novel gene, MMSET, resulting in IgH/MMSET hybrid transcripts. *Blood*. 1998;92:3025-3034.
95. Marango J, Shimoyama M, Nishio H, et al. The MMSET protein is a histone methyltransferase with characteristics of a transcriptional corepressor. *Blood*. 2008;111:3145-3154.
96. Martinez-Garcia E, Popovic R, Min DJ, et al. The MMSET histone methyl transferase switches global histone methylation and alters gene expression in t(4;14) multiple myeloma cells. *Blood*. 2011;117:211-220.
97. Todoerti K, Ronchetti D, Agnelli L, et al. Transcription repression activity is associated with the type I isoform of the MMSET gene involved in t(4;14) in multiple myeloma. *Br J Haematol*. 2005;131:214-218.
98. Kim JY, Kee HJ, Choe NW, et al. Multiple-myeloma-related WHSC1/MMSET isoform RE-IIBP is a histone methyltransferase with transcriptional repression activity. *Mol Cell Biol*. 2008;28:2023-2034.
99. Walker BA, Wardell CP, Chiecchio L, et al. Aberrant global methylation patterns affect the molecular pathogenesis and prognosis of multiple myeloma. *Blood*. 2011;117:553-562.
100. Pei H, Zhang L, Luo K, et al. MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature*. 2011;470:124-128.
101. Rosati R, La Starza R, Veronese A, et al. NUP98 is fused to the NSD3 gene in acute myeloid leukemia associated with t(8;11)(p11.2;p15). *Blood*. 2002;99:3857-3860.
102. Angrand PO, Apiou F, Stewart AF, et al. NSD3, a new SET domain-containing gene, maps to 8p12 and is amplified in human breast cancer cell lines. *Genomics*. 2001;74:79-88.
103. Tonon G, Wong KK, Maulik G, et al. High-resolution genomic profiles of human lung cancer. *Proc Natl Acad Sci U S A*. 2005;102:9625-9630.
104. Jaju RJ, Fidler C, Haas OA, et al. A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. *Blood*. 2001;98:1264-1267.
105. van Haaften G, Dalgliesh GL, Davies H, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. *Nat Genet*. 2009;41:521-523.
106. Smith ER, Lee MG, Winter B, et al. Drosophila UTX is a histone H3 Lys27 demethylase that colocalizes with the elongating form of RNA polymerase II. *Mol Cell Biol*. 2008;28:1041-1046.
107. Chang CJ, Yang JY, Xia W, et al. EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. *Cancer Cell*. 2011;19:86-100.
108. Varambally S, Cao Q, Mani RS, et al. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science*. 2008;322:1695-1699.
109. Zhan F, Hardin J, Kordsmeier B, et al. Global gene expression profiling of multiple myeloma, monoclonal gammopathy of undetermined significance, and normal bone marrow plasma cells. *Blood*. 2002;99:1745-1757.
110. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci U S A*. 2010;107:20980-20985.
111. Yap DB, Chu J, Berg T, et al. Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. *Blood*. 2011;117:2451-2459.
112. Croonquist PA, Van Ness B. The polycomb group protein enhancer of zeste homolog 2 (EZH 2) is an oncogene that influences myeloma cell growth and the mutant ras phenotype. *Oncogene*. 2005;24:6269-6280.
113. Wang JK, Tsai MC, Poulin G, et al. The histone demethylase UTX enables RB-dependent cell fate control. *Genes Dev*. 2010;24:327-332.
114. Agger K, Cloos PA, Christensen J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature*. 2007;449:731-734.
115. Hudlebusch HR, Lodahl M, Johnsen HE, et al. Expression of HOXA genes in patients with multiple myeloma. *Leuk Lymphoma*. 2004;45:1215-1217.
116. Elnenaei MO, Gruszka-Westwood AM, A'Hernt R, et al. Gene abnormalities in multiple myeloma: the relevance of TP53, MDM2, and CDKN2A. *Haematologica*. 2003;88:529-537.
117. Ng MH, Chung YF, Lo KW, et al. Frequent hypermethylation of p16 and p15 genes in multiple myeloma. *Blood*. 1997;89:2500-2506.
118. Gonzalez-Paz N, Chng WJ, McClure RF, et al. Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. *Blood*. 2007;109:1228-1232.
119. Dib A, Barlogie B, Shaughnessy Jr JD, et al. Methylation and expression of the p16INK4A tumor suppressor gene in multiple myeloma. *Blood*. 2007;109:1337-1338.
120. Ribas C, Colleoni GW, Felix RS, et al. p16 gene methylation lacks correlation with angiogenesis and prognosis in multiple myeloma. *Cancer Lett*. 2005;222:247-254.
121. Kramer A, Schultheis B, Bergmann J, et al. Alterations of the cyclin D1/pRb/p16(INK4A) pathway in multiple myeloma. *Leukemia*. 2002;16:1844-1851.
122. Dib A, Peterson TR, Raducha-Grace L, et al. Paradoxical expression of INK4c in proliferative multiple myeloma tumors: bi-allelic deletion vs increased expression. *Cell Div*. 2006;1:23.
123. Tasaka T, Berenson J, Vescio R, et al. Analysis of the p16INK4A, p15INK4B and p18INK4C genes in multiple myeloma. *Br J Haematol*. 1997;96:98-102.
124. Boyd KD, Ross FM, Walker BA, et al. Mapping of chromosome 1p deletions in myeloma identifies FAM46C at 1p12 and CDKN2C at 1p32.3 as being genes in regions associated with adverse survival. *Clin Cancer Res*. 2011;17:7776-7784.
125. Demchenko YN, Glebov OK, Zingone A, et al. Classical and/or alternative NFkappaB pathway activation in multiple myeloma. *Blood*. 2010;115:3541-3552.
126. Neri A, Baldini L, Trecca D, et al. p53 gene mutations in multiple myeloma are associated with advanced forms of malignancy. *Blood*. 1993;81:128-135.
127. Preudhomme C, Facon T, Zandecki M, et al. Rare occurrence of P53 gene mutations in multiple myeloma. *Br J Haematol*. 1992;81:440-443.
128. Portier M, Moles JP, Mazars GR, et al. p53 and RAS gene mutations in multiple myeloma. *Oncogene*. 1992;7:2539-2543.
129. Corradini P, Inghirami G, Astolfi M, et al. Inactivation of tumor suppressor genes, p53 and Rb1, in plasma cell dyscrasias. *Leukemia*. 1994;8:758-767.
130. Mazars GR, Portier M, Zhang XG, et al. Mutations of the p53 gene in human myeloma cell lines. *Oncogene*. 1992;7:1015-1018.
131. Chng WJ, Price-Troska T, Gonzalez-Paz N, et al. Clinical significance of TP53 mutation in myeloma. *Leukemia*. 2007;21:582-584.
132. Avet-Loiseau H, Li JY, Godon C, et al. P53 deletion is not a frequent event in multiple myeloma. *Br J Haematol*. 1999;106:717-719.
133. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. *Blood*. 2007;109:3489-3495.
134. Chang H, Qi C, Yi QL, et al. p53 gene deletion detected by fluorescence in situ hybridization is an adverse prognostic factor for patients with multiple myeloma following autologous stem cell transplantation. *Blood*. 2005;105:358-360.
135. Gertz MA, Lacy MQ, Dispenzieri A, et al. Clinical implications of t(11;14)(q13;q32), t(4;14)(p16.3;q32), and -17p13 in myeloma patients treated with high-dose therapy. *Blood*. 2005;106:2837-2840.
136. Davies FE, Dring AM, Li C, et al. Insights into the multistep transformation of MGUS to myeloma using microarray expression analysis. *Blood*. 2003;102:4504-4511.
137. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res*. 2001;61:3071-3076.
138. Kaufmann H, Ackermann J, Baldia C, et al. Both IGH translocations and chromosome 13q deletions are early events in monoclonal gammopathy of undetermined significance and do not evolve during transition to multiple myeloma. *Leukemia*. 2004;18:1879-1882.

139. Hardin J, Waddell M, Page CD, et al. Evaluation of multiple models to distinguish closely related forms of disease using DNA microarray data: an application to multiple myeloma. *Stat Appl Genet Mol Biol*. 2004;3(10).
140. Zhan F, Barlogie B, Arzoumanian V, et al. Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. *Blood*. 2007;109:1692-1700.
141. Perez-Persona E, Vidriales MB, Mateo G, et al. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. *Blood*. 2007;110:2586-2592.
142. Rajkumar SV, Kyle RA, Therneau TM, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. *Blood*. 2005;106:812-817.
143. Paquette RL, Berenson J, Lichtenstein A, et al. Oncogenes in multiple myeloma: point mutation of N-ras. *Oncogene*. 1990;5:1659-1663.
144. Neri A, Murphy JP, Cro L, et al. Ras oncogene mutation in multiple myeloma. *J Exp Med*. 1989;170:1715-1725.
145. Bezieau S, Devilder MC, Avet-Loiseau H, et al. High incidence of N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. *Hum Mutat*. 2001;18:212-224.
146. Liu P, Leong T, Quam L, et al. Activating mutations of N- and K-ras in multiple myeloma show different clinical associations: analysis of the Eastern Cooperative Oncology Group Phase III Trial. *Blood*. 1996;88:2699-2706.
147. Corradini P, Ladetto M, Voena C, et al. Mutational activation of N- and K-ras oncogenes in plasma cell dyscrasias. *Blood*. 1993;81:2708-2713.
148. Rasmussen T, Kuehl M, Lodahl M, et al. Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors. *Blood*. 2005;105:317-323.
149. Keats JJ, Reiman T, Maxwell CA, et al. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. *Blood*. 2003;101:1520-1529.
150. Malgeri U, Baldini L, Perfetti V, et al. Detection of t(4;14)(p16.3;q32) chromosomal translocation in multiple myeloma by reverse transcription-polymerase chain reaction analysis of IGH-MMSET fusion transcripts. *Cancer Res*. 2000;60:4058-4061.
151. Egan JB, Shi CX, Tembe W, et al. Whole-genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution, and clonal tides. *Blood*. 2012;120:1060-1066.
152. Keats JJ, Chesi M, Egan JB, et al. Clonal competition with alternating dominance in multiple myeloma. *Blood*. 2012;120:1067-1076.

Mario D. Terán, Ronan Kelly, and Malcolm V. Brock

31

Molecular Mechanisms of Esophageal Cancer

Introduction

The cancers arising from the esophageal mucosa, primarily esophageal adenocarcinoma (EAC) and esophageal squamous-cell carcinoma (ESCC), are clinically some of the most devastating and lethal cancers in the world. Collectively, they are the eighth most common cancer diagnosed worldwide, with approximately 482,300 new cases in 2008.[1] International age-adjusted incidence rates vary drastically, with a nearly 16-fold difference between the high-incidence areas of southern and eastern Africa and eastern Asia and the low-incidence areas of middle and western Africa and Central America.[1]

Worldwide, ESCC accounts for the majority of esophageal cancers diagnosed, because of its high prevalence in the "esophageal cancer belt," an area stretching from central Asia to north and central China (Figure 31-1). In this high-risk area, 90% of all esophageal cancers are SCC, with as many as 120 to 175 new cases per 100,000 inhabitants each year.[1-3] However, the incidence rates of EAC and ESCC vary drastically among geographical regions. In the Western world, EAC has overtaken ESCC as the most prevalent form of esophageal cancer.[3,4] The United States alone has seen a fourfold increase in EAC since the 1970s.[5]

Regardless of the histology, esophageal cancer carries a high mortality rate because of its advanced stage of presentation, with an overall 5-year survival rate of 17%.[4] It is the sixth most common cause of cancer death worldwide, claiming 4,070,800 lives in 2008.[1,2] Approximately 86% of these deaths occurred in developing nations,[1,2] where more than 90% of the esophageal cancers diagnosed were stage 2 or greater.[6]

Histology

Ninety-five percent of esophageal cancers are of epithelial origin, occurring as either EAC or SCC, with the remaining tumor histologies comprising malignant melanoma, lymphoma, carcinoid, small cell carcinoma, or sarcoma.[7] Esophageal adenocarcinomas primarily occur in the distal one third of the esophagus and develop through a progressive malignant sequence beginning with cellular metaplasia, then low- and high-grade dysplasia, and eventually to invasive adenocarcinoma.[8] Esophageal squamous-cell carcinoma primarily emerges in the middle to upper third of the esophagus and follows a similar stepwise developmental sequence. However, the initiating histological lesion in the squamous histology is mild to severe dysplasia, followed by carcinoma in situ, and finally invasive squamous-cell carcinoma.[9]

Etiology and Molecular Mechanisms of Esophageal Cancer

Esophageal adenocarcinoma and squamous-cell carcinoma are two distinct cancers that differ not only in histology and geographical distribution, but also in the risk factors that contribute to their development. However, their risk factors stem from similar pathological processes and also similar molecular mechanisms that lead to tumorigenesis.[5,10] The pathological processes that initiate these tumorigenic mechanisms stem from chronic irritation and inflammation, carcinogenic exposures, or a combination of both. Unique risk factors of both EAC and ESCC will be highlighted in the context of either chronic inflammation or carcinogenic exposure. More detailed explanations of the epidemiological risk factors associated with EAC and ESCC are covered elsewhere in this text. Simply stated, examples of chronic inflammation can take the form of gastroesophageal reflux as in EAC, or chronic thermal injury of the more proximal esophagus from drinking hot beverages in ESCC. Similarly, carcinogenic exposure can include the high prevalence of tobacco smoking in both EAC and ESCC as well as the high nitrosamine content of

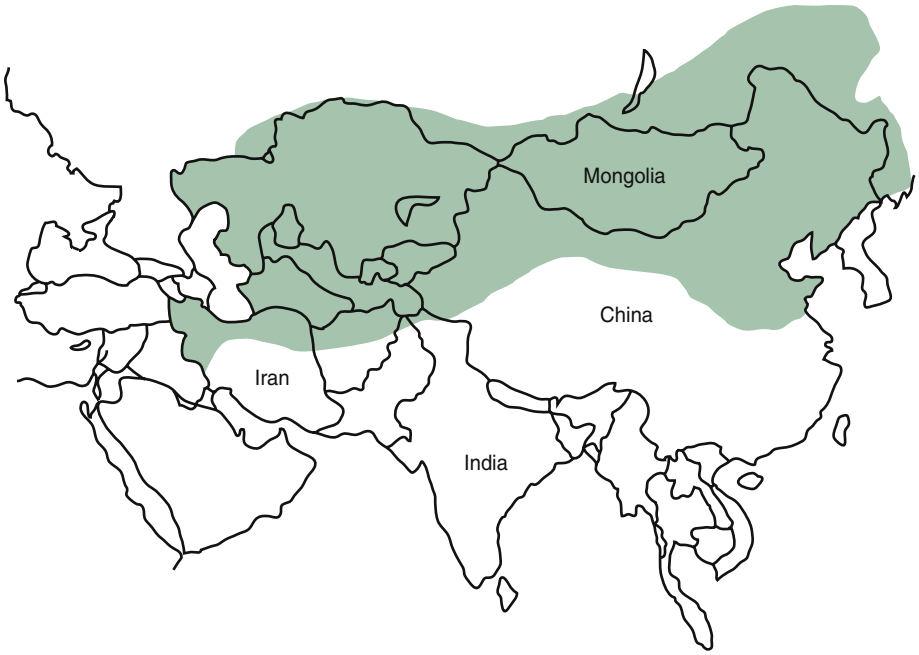


Figure 31-1 The central Asian esophageal cancer belt extending from Iran to China. *Modified from Kamangar F, Malekzadeh R, Dawsey SM, et al. Esophageal cancer in northeastern Iran: a review.* Arch Iranian Med. *2007;10:70-82.*

the soil and the high consumption of smoked foods in the esophageal cancer belt in ESCC.

Chronic Inflammation and Esophageal Cancer

The inflammatory response is a highly complex and coordinated system to promote cellular regeneration and proliferation. This response provides an environment rich in inflammatory cells, growth factors, adhesion molecules, and angiogenic mediators, all of which can potentiate and initiate tumorigenesis.[11] Rudolf Virchow first postulated a possible link between tumorigenesis and the inflammatory system in the 19th century, when he observed the presence of leukocytes within tumors.[12] Only recently, however, have many of the underlying molecular mechanisms between inflammation and cancer initiation been elucidated[11,13,14] (Figure 31-2).

Nuclear Factor-Kappa B

Nuclear factor-kappa B (NFκB) is a pro-inflammatory transcription factor initially described as a B-cell–specific factor for the immunoglobulin-κ light chain gene.[15] As part of the Rel protein family of transcription factors, NFκB exists in the cytoplasm as an inactive dimer until activated by a diverse set of extracellular stimuli and signals, such as inflammatory cytokines and growth factors.[16,17] These stimuli trigger a common pathway of phosphorylation, ubiquitination, and proteasome-dependent degradation of NFκB's regulatory protein, inhibitor of NFκB (IκB), to activate NFκB.[17] Once activated, NFκB is quickly translocated into the nucleus and binds to promoter regions responsible for the transcription of genes that encode for multiple cytokines (IL6, IL8, TNFα), cellular adhesion molecules (ICAM1, E-selectin), cell cycle regulators (p21, cyclin D1), apoptosis regulators (surviving, Bcl-2), and other transcription factors (p53, c-myc).[13,16,17]

Aberrant activation of NFκB has been implicated in the initiation and progression of many cancers, including esophageal cancer, because of its broad role in inflammation, apoptosis, and cell survival[13] (Figure 31-3). Barrett's esophagus (BE), as the main risk factor, is due to chronic epithelial damage from gastroesophageal bile and acid reflux.[22] NFκB activation has been described as a central event in the development of BE, and thus in EAC's initiation and progression.[18,19] A 2004 study was the first to show that NFκB expression was increased in the epithelial cells of BE compared to normal esophageal epithelial cells.[18] In addition, this study revealed that 61% of resected EAC tumors displayed NFκB immunoreactivity, and NFκB-positive tumors were more likely to be of advanced stage.[21] Others have also validated these data[19] and have even identified NFκB as an emerging molecular prognostic marker: NFκB tumor positivity is correlated with chemoradiation resistance and poor outcomes.[18,20,21]

Although BE and gastroesophageal reflux are well-documented risk factors for EAC, the molecular pathogenic correlation between risk factors and the development of esophageal squamous-cell carcinoma is less clear.[5,9,22]

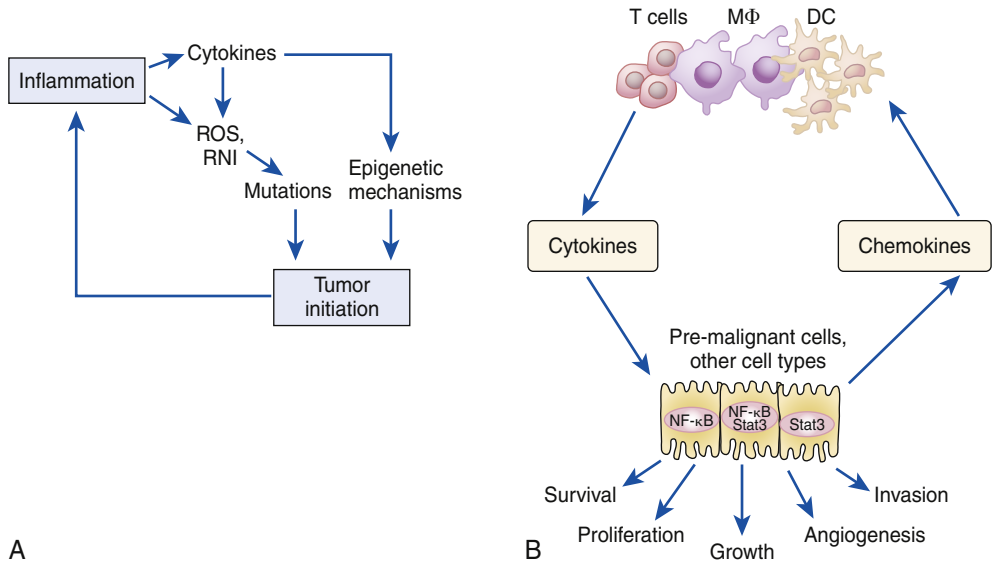


Figure 31-2 Role of inflammation in tumor initiation and promotion **(A)** Tumor initiation. Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced by inflammatory cells may cause mutations in neighboring epithelial cells. Also, cytokines produced by inflammatory cells can elevate intracellular ROS and RNI in premalignant cells. In addition, inflammation can result in epigenetic changes that favor tumor initiation. Tumor-associated inflammation contributes to further ROS, RNI, and cytokine production. **(B)** Tumor promotion. Cytokines produced by tumor infiltrating immune cells activate key transcription factors, such as NFκB or STAT3, in premalignant cells to control numerous pro-tumorigenic processes, including survival, proliferation, growth, angiogenesis, and invasion. As parts of positive feed-forward loops, NFκB and STAT3 induce production of chemokines that attract additional immune/inflammatory cells to sustain tumor-associated inflammation. *Modified from Grivennikov S, Greten FR, Karin M. Immunity, inflammation, and cancer.* Cell. *2010;140:883-899.*

The mechanism for epithelial injury is quite different from that in adenocarcinoma and is usually due to thermal injury from hot liquids and foods, corrosive irritation from retained esophageal contents due to achalasia, or caustic ingestion of corrosive agents.[23-25] Nonetheless, abnormal activation of NFκB has also been linked to ESCC. In vitro models have shown that NFκB pathways are highly expressed in esophageal SCC cell lines.[26] Hatata and colleagues found that NFκB was overexpressed in 61% of ESCC resected tumors.[27] They also reported that patients with NFκB positivity in their tumors correlated with significantly poorer survival times when compared to patients with NFκB negative-staining neoplasms (15 versus 42 months, $P = .007$).

Activation of Additional Inflammatory Mediators and Esophageal Cancer

Abnormal activation of downstream NFκB targets, such as COX-2 and IL6, has also been implicated independently in the pathogenesis of esophageal cancer. Cyclooxygenase-2 (COX-2), is one of two isozymes that catalyze the rate-limiting conversion of arachidonic acid to prostaglandins, prostacyclins, and thromboxanes.[28] COX-2 protein expression has been found to be significantly increased in patient samples with BE and EAC when compared to normal esophageal tissue.[29] In addition, bile acid exposure to esophageal cells has been shown significantly to induce COX-2 expression.[29] COX-2 overexpression has also been found in ESCC tumors, specifically well-differentiated tumors, and its progressive expression correlates well with advancing clinical stage.[30] Although in vitro investigations into selective COX-2 inhibition for the treatment of EAC and ESCC have shown some promise,[31,32] clinical trials have yet to demonstrate efficacy.[33,34]

Interleukin-6 (IL6) is a multifunctional cytokine that has a range of functions from acute-phase protein induction to cellular growth and differentiation.[35] Once bound to its extracellular receptor (IL6R), IL6 induces gene transcription through two signaling pathways, the JAK-STAT (Janus family tyrosine kinase-signal transducer and activator of transcription) pathway and the Ras-MAPK (mitogen-activated protein kinase) pathway[36,37] (Figure 31-4). Like NFκB and COX-2, IL6 has been demonstrated to be a key mediator in the metaplastic conversion of normal esophageal squamous epithelium to BE as well as the further dysplastic conversion to EAC.[38,39] Because of its multifunctionality and production by multiple cells throughout the body, IL6 is readily detected in the serum and has shown promise as a noninvasive diagnostic tumor marker. In 2011, Lukaszewicz-Zajac and colleagues demonstrated that when compared to traditional esophageal cancer tumor markers of carcinoembryonic antigen (CEA) and squamous-cell cancer antigen (SCC-Ag), elevated IL6 serum concentrations correlated better with early esophageal cancer diagnosis.[40] Overall, the elevated serum IL6 concentrations could be detected in

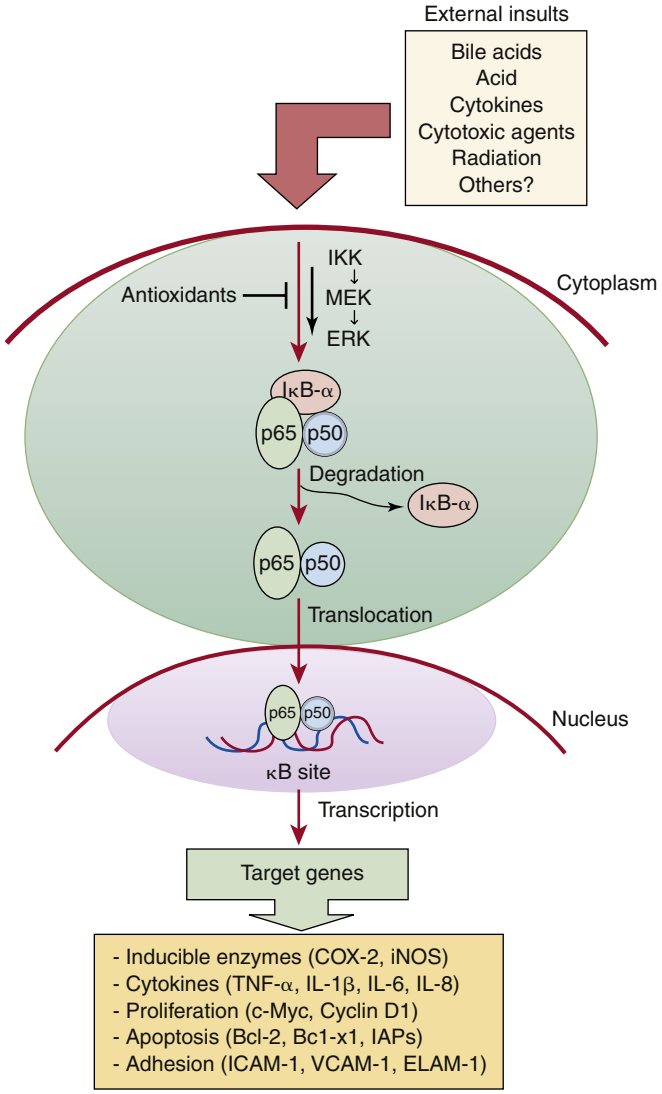


Figure 31-3 Diagram showing activation of NFκB and target genes in esophageal cancer. *Modified from Abdel-Latif MM, Dermot Kelleher K, Reynolds JV. Potential role of NF-κB in esophageal adenocarcinoma: as an emerging molecular target.* J Surg Res. *2009;153:172-180.*

either EAC or ESCC with 87% sensitivity and 92% specificity with a statistically significant area under the receiver operating characteristic curve of 0.92 when compared to either CEA or SCC-Ag.[40]

Environmental Carcinogenic Exposures and Esophageal Cancer

The link between certain environmental carcinogens, such as tobacco smoking, and tumorigenesis has been well established for many cancers. The ability of these carcinogens to promote tumorigenesis stems from their abilities to cause genomic aberrations leading to unregulated and abnormal cellular growth.[41] Both EAC and ESCC have many unique genomic and epigenomic abnormalities that have been identified and studied.[10] However, EAC and ESCC have been found to have similar aberrant genomic changes in cell-cycle regulator genes, such as p53, APC, RB, and cyclin-D1, early in their tumorigenesis[42-44] (Figure 31-5). Specific environmental risk factors for esophageal cancer and their molecular pathogenesis are now being revealed.

Tobacco Smoking

The association between tobacco smoking and lung cancer has long been recognized as due to the abundance of carcinogens in tobacco smoke. The International Agency for Research on Cancer has identified more than 60 substances in cigarette smoke for which sufficient evidence exists for carcinogenicity in either laboratory animals or humans.[45] Tobacco smoking is one of the few risk factors shared by both EAC and ESCC. In a study of 474,606 participants, current and former smokers had a significantly increased risk of developing EAC when compared to never-smokers (hazard ratio [HR] 3.7; 95% confidence interval [CI] 2.2-6.2 and 2.8; 95% CI 1.8-4.3, respectively).[46] The same study also revealed that current smokers were at an even higher risk for developing ESCC (HR 9.2; 95% CI 4.0-21.3), and individuals who had ever smoked accounted for 77% of ESCC cases.[46]

Two of the most potent and well-studied tobacco smoke carcinogens are polyaromatic hydrocarbons (PAHs) and the tobacco-specific *N*-nitrosamine, nicotine-derived nitrosamine ketone (NNK). Their tumorigenic effects arise from their ability to form DNA adducts as well as intra- and interstrand DNA crosslinks.[47] The proteins of the nucleotide excision repair pathway and specialized DNA polymerases repair and bypass various types of DNA damage acquired by PAHs or NNK.[48,49] However, because of the constant exposure to PAHs and NNK with chronic smoking, the amount of DNA damage done can overwhelm the DNA repair system, resulting in chromosomal aberrations, halted DNA replication, and mutations.[47] The resulting alterations may lead to abnormal cellular growth and transformation if proto-oncogenes or tumor suppressor genes are affected.

Alcohol

Chronic alcohol abuse is an important risk factor in the development of ESCC, but has no correlation with increased EAC risk.[46] Heavy drinkers (more than 84 drinks per week) have nearly a 25-fold increase in ESCC risk compared to light drinkers (1 to 20 drinks per week).[50] In addition, exposure to both tobacco and alcohol has been found to have a synergistic effect on the risk of developing ESCC.[51]

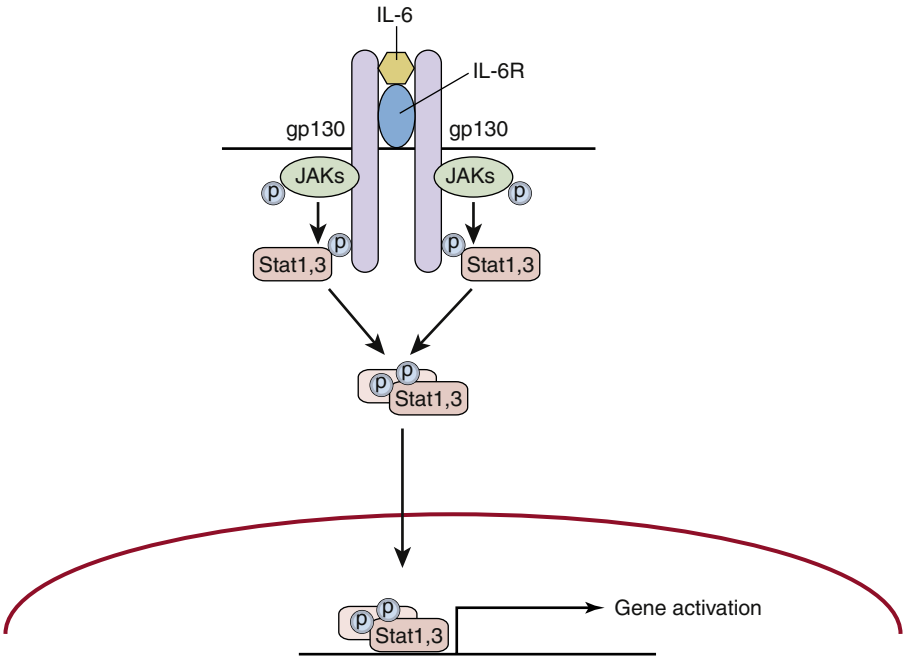


Figure 31-4 IL6 signal transduction IL6-mediated stimulation induces homodimerization of gp130, activating (phosphorylating) JAKs, and Stat1 and Stat3. Activated Stat1 and Stat3 form homodimers or heterodimers, which induce activation of various genes. *Modified from Kishimoto T. IL-6: from its discovery to clinical applications.* Int Immunol. *2010;22:347-352.*

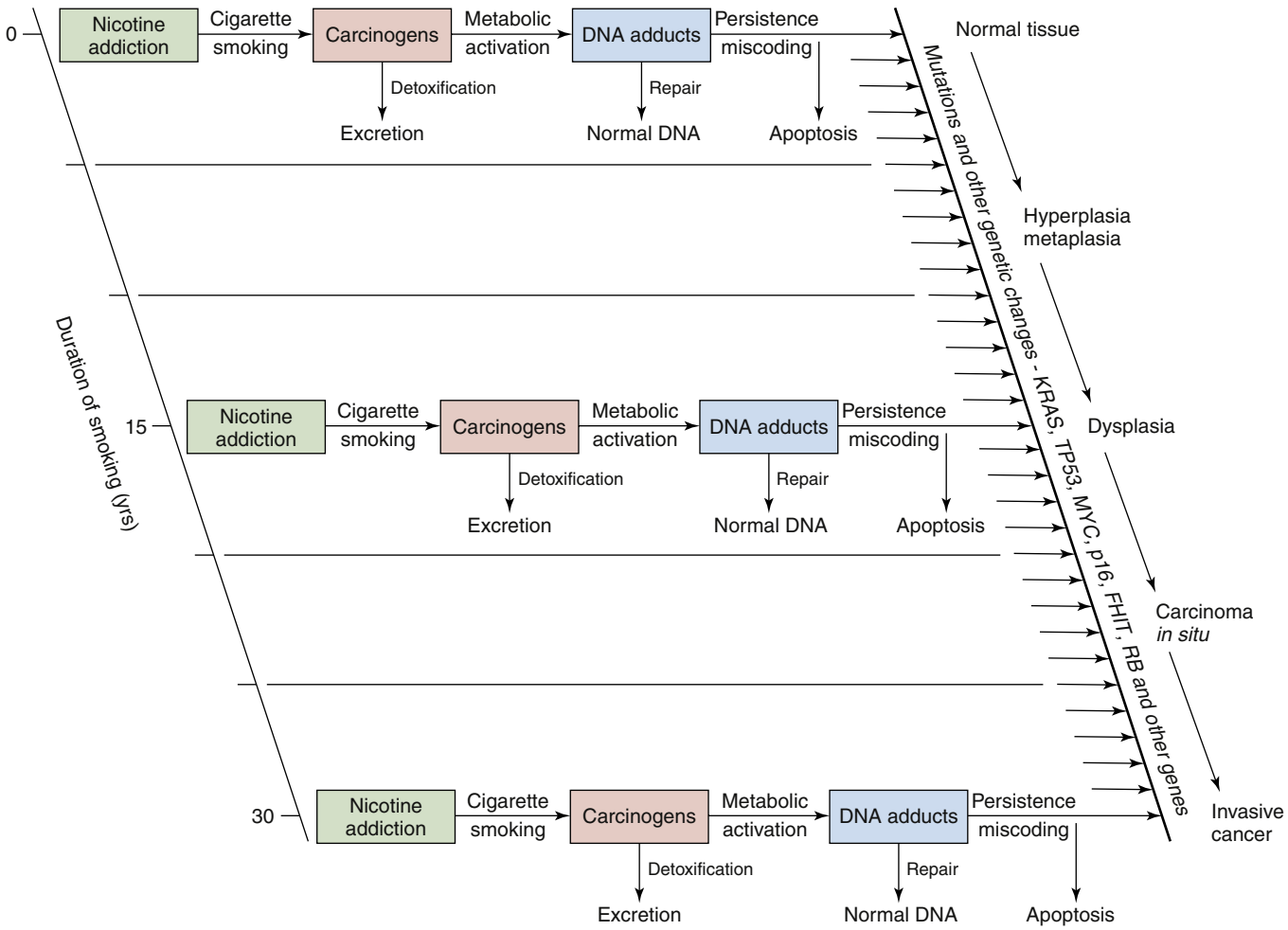


Figure 31-5 Scheme linking cigarette-smoke carcinogens with multiple genetic changes in lung cancer A key aspect is the chronic exposure of DNA to multiple metabolically activated carcinogens, leading to multiple adducts and their consequent mutations. The time period and sequence of genetic events are uncertain. *Modified from Pfeifer GP, Denissenko MF, Olivier M, et al. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers.* Oncogene. *2002;21:7435-7451.*

The pathophysiology underlying alcohol's role in tumorigenesis of ESCC involves esophageal irritation, through pathways previously described, and its principal metabolite, acetaldehyde, a known carcinogen causing DNA adducts.[52] The primary enzymes responsible for metabolizing acetaldehyde into an inert metabolite are aldehyde dehydrogenase-2 (ALDH2) and alcohol dehydrogenase-1B (ADH1B). ALDH2 and ADH1B are both present in the mouth, and mutations in either of these genes create increased salivary acetaldehyde concentrations, providing direct carcinogenic exposure to the esophageal mucosa.[53,54] For example, homozygotes for the ALDH2 gene have been shown to have acetaldehyde levels up to 13 times greater than normal individuals.[53] ALDH2 and ADH1B mutations are rare in Western populations; however, they are very prevalent in eastern Asian populations and are thought to contribute to the high incidence of ESCC in the esophageal cancer belt.[53,55,56]

Diet

Diets high in fruit and vegetable intake have been shown to be protective against many cancers because of the high concentrations of anticarcinogenic compounds.[57] Many dietary studies have demonstrated that diets low in fruit and vegetables have an increased risk of ESCC.[58,59] Dietary intake of nitrogen- and nitrosamine-rich foods have also been associated with ESCC development, especially in the high-risk areas of the esophageal cancer belt where nitrogen-rich foods and water are consumed in high concentrations.[59-62] In the Hebei province in China, the highest incidence areas of ESCC correlated with the highest concentrations of nitrite and ammonia nitrogen in drinking water.[59] In addition, in many of these areas cultural customs lead to widespread consumption of hot beverages, which was vital in establishing the connection between thermal irritation and esophageal cancer.[63,64]

Key References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.
3. Curado MP, Edwards B, Shin HR, et al. *Cancer Incidence in Five Continents*. Vol 9. IARC Scientific Publication No 160; 2009.
4. Howlader N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations)*. Bethesda, Md: National Cancer Institute. Retrieved from http://seer.cancer.gov/csr/1975_2009_pops09.
5. Holmes RS, Vaughan TL. Epidemiology and pathogenesis of esophageal cancer. *Semin Radiat Oncol*. 2007;17:2-9.
6. Abdullah M, Karim AA, Goh KL. Late presentation of esophageal cancer: observations in a multiracial South-East Asian population. *J Dig Dis*. 2010;11:28-33.
7. Das A. Tumors of the esophagus. In: Feldman M, Friedman LS, Brandt LJ, eds. *Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management*. 9th ed. Philadelphia, Pa: WB Saunders; 2010:745-770.
9. Wang GQ, Abnet CC, Shen Q, et al. Histological precursors of oesophageal squamous cell carcinoma: results from a 13 year prospective follow up study in a high risk population. *Gut*. 2005;54:187-192.
10. Kwong KF. Molecular biology of esophageal cancer in the genomics era. *Surg Clin North Am*. 2005;85:539-553.
12. Heidland A, Klassen A, Rutkowski P, et al. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? *J Nephrol*. 2006;19:S102-S109.
13. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441:431-436.
14. Grivennikov S, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140:883-899.
18. Abdel-Latif MM, Dermot Kelleher K, Reynolds JV. Potential role of NF-κB in esophageal adenocarcinoma: as an emerging molecular target. *J Surg Res*. 2009;153:172-180.
19. Konturek PC, Nikiforuk A, Kania J, et al. Activation of NFκB represents the central event in the neoplastic progression associated with Barrett's esophagus: a possible link to the inflammation and overexpression of COX-2, PPARγ, and growth factors. *Dig Dis Sci*. 2004;49:1075-1083.
20. Izzo JG, Malhotra U, Wu TT, et al. Association of activated transcription factor nuclear factor κB with chemoradiation resistance and poor outcome in esophageal carcinoma. *J Clin Oncol*. 2006;24:748-754.
21. Abdel-Latif MM, O'Riordan J, Windle HJ, et al. NF-κB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann Surg*. 2004;239:491-500.
22. Hvid-Jensen F, Pedersen L, Drewes AM, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med*. 2011;365:1375-1383.
24. Islami F, Boffetta P, Ren JS, et al. High-temperature beverages and foods and esophageal cancer risk-a systematic review. *Int J Cancer*. 2009;125:491-524.
25. Ruol A, Rampado S, Parenti A, et al. Caustic ingestion and oesophageal cancer: intra- and peri-tumoral fibrosis is associated with a better prognosis. *Eur J Cardiothorac Surg*. 2010;38:659-664.
26. Tian F, Zang WD, Hou WH, et al. Nuclear factor-κB signaling pathway constitutively activated in esophageal squamous cell carcinoma cell lines and inhibition of growth of cells by small interfering RNA. *Acta Biochim Biophys Sin*. 2006;38:318-326.
27. Hatata T, Higaki K, Tatebe S, et al. Immunohistochemical study of nuclear factor-kB expression in esophageal squamous cell carcinoma: prognostic significance and sensitivity to treatment with 5-FU. *Dis Esophagus*. 2012;25:716-722.
30. Yang GZ, Li L, Ding HY, et al. Cyclo-oxygenase-2 is overexpressed in Chinese esophageal squamous cell carcinoma, and correlated with NF-κB: an immunohistochemical study. *Exp Mol Pathol*. 2005;79:214-218.
31. Souza RH, Shewmake K, Beer DG, et al. Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. *Cancer Res*. 2000;60:5767-5772.
32. Liu X, Li P, Zhang ST, et al. COX-2 mRNA expression in esophageal squamous cell carcinoma (ESCC) and effect by NSAID. *Dis Esophagus*. 2008;21:9-14.
33. Liu JF, Jamieson GG, Wu TC, et al. A preliminary study on the postoperative survival of patients given aspirin after resection for squamous cell carcinoma of the esophagus or adenocarcinoma of the cardia. *Ann Surg Oncol*. 2009;16:1397-1402.
34. Mantraradi RVP, Fineberg N, Ansari R, et al. A phase II study of cisplatin, 5 fluorouracil (5-FU), radiation (RT) and celecoxib in patients with resectable esophageal cancer (EC): updated results from the Hoosier Oncology Group (HOG) study. *J Clin Oncol*. 2004;22:4052.
35. Kishimoto T. IL-6: from its discovery to clinical applications. *Int Immunol*. 2010;22:347-352.
36. Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol*. 2007;178:2623-2629.

38. Dvorakova K, Payne CM, Ramsey L, et al. Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. *Clin Cancer Res*. 2004;10:2020-2028.
39. Quante M, Bhagat G, Abrams J, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett's-like metaplasia. *Cancer Cell*. 2012;21:36-51.
40. Lukaszewicz-Zajac M, Mroczko B, Kozlowski M, et al. Higher importance of interleukin 6 than classic tumor markers (carcinoembryonic antigen and squamous cell cancer antigen) in the diagnosis of esophageal cancer patients. *Dis Esophagus*. 2012;25:242-249.
46. Freedman ND, Abnet CC, Leitzmann MF, et al. A prospective study of tobacco, alcohol, and the risk of esophageal and gastric cancer subtypes. *Am J Epidemiol*. 2007;165:1424-1433.
51. Lee CH, Wu DC, Lee JM, et al. Carcinogenetic impact of alcohol intake on squamous cell carcinoma risk of the oesophagus in relation to tobacco smoking. *Eur J Cancer*. 2007;43:1188-1199.
52. Salaspuro M. Acetaldehyde: a cumulative carcinogen in humans. *Addiction*. 2009;104:551-553.
53. Hiyama T, Yoshihara M, Tanaka S, et al. Genetic polymorphisms and esophageal cancer risk. *Int J Cancer*. 2007;121:1643-1658.
55. Yang SJ, Wang HY, Li XQ, et al. Genetic polymorphisms of ADH2 and ALDH2 association with esophageal cancer risk in southwest China. *World J Gastroenterol*. 2007;13:5760-5764.
57. Riboli E, Norat T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr*. 2003;78:559-569.
58. Freedman ND, Park Y, Subar AF, et al. Fruit and vegetable intake and esophageal cancer in a large prospective cohort study. *Int J Cancer*. 2007; 121:2753-2760.
60. Jakszyn P, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. *World J Gastroenterol*. 2006;12:4296-4303.
61. Hotchkiss JH. Preformed N-nitroso compounds in foods and beverages. *Cancer Surv*. 1989;8:295-321.

This page intentionally left blank

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.
3. Curado MP, Edwards B, Shin HR, et al. *Cancer Incidence in Five Continents*. Vol 9. IARC Scientific Publication No 160; 2009.
4. Howlader N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975-2009 (Vintage 2009 Populations)*. Bethesda, Md: National Cancer Institute. Retrieved from http://seer.cancer.gov/csr/1975_2009_pops09.
5. Holmes RS, Vaughan TL. Epidemiology and pathogenesis of esophageal cancer. *Semin Radiat Oncol*. 2007;17:2-9.
6. Abdullah M, Karim AA, Goh KL. Late presentation of esophageal cancer: observations in a multiracial South-East Asian population. *J Dig Dis*. 2010;11:28-33.
7. Das A. Tumors of the esophagus. In: Feldman M, Friedman LS, Brandt LJ, eds. *Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management*. 9th ed. Philadelphia, Pa: WB Saunders; 2010:745-770.
8. Jankowski JA, Wright NA, Meltzer SJ, et al. Molecular evolution of the metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Pathol*. 1999;154:965-973.
9. Wang GQ, Abnet CC, Shen Q, et al. Histological precursors of oesophageal squamous cell carcinoma: results from a 13 year prospective follow up study in a high risk population. *Gut*. 2005;54:187-192.
10. Kwong KF. Molecular biology of esophageal cancer in the genomics era. *Surg Clin North Am*. 2005;85:539-553.
11. Coussens L, Werb Z. Inflammation and cancer. *Nature*. 2002;420:860-867.
12. Heidland A, Klassen A, Rutkowski P, et al. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? *J Nephrol*. 2006;19:S102-S109.
13. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441:431-436.
14. Grivennikov S, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140:883-899.
15. Sen R, Baltimore D. Inducibility of κ immunoglobulin enhancer binding protein NF-κB by a post-translational mechanism. *Cell*. 1986;47:921.
16. Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. *Annu Rev Immunol*. 1994;12:141-179.
17. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene*. 1999;18:6853-6866.
18. Abdel-Latif MM, Dermot Kelleher K, Reynolds JV. Potential role of NF-κB in esophageal adenocarcinoma: as an emerging molecular target. *J Surg Res*. 2009;153:172-180.
19. Konturek PC, Nikiforuk A, Kania J, et al. Activation of NFκB represents the central event in the neoplastic progression associated with Barrett's esophagus: a possible link to the inflammation and overexpression of COX-2, PPARγ, and growth factors. *Dig Dis Sci*. 2004;49:1075-1083.
20. Izzo JG, Malhotra U, Wu TT, et al. Association of activated transcription factor nuclear factor κB with chemoradiation resistance and poor outcome in esophageal carcinoma. *J Clin Oncol*. 2006;24:748-754.
21. Abdel-Latif MM, O'Riordan J, Windle HJ, et al. NF-κB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann Surg*. 2004;239:491-500.
22. Hvid-Jensen F, Pedersen L, Drewes AM, et al. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med*. 2011;365:1375-1383.
23. Sandler RS, Nyren O, Ekbom A, et al. The risk of esophageal cancer in patients with achalasia: a population-based study. *JAMA*. 1995;274:1359-1362.
24. Islami F, Boffetta P, Ren JS, et al. High-temperature beverages and foods and esophageal cancer risk-a systematic review. *Int J Cancer*. 2009;125:491-524.
25. Ruol A, Rampado S, Parenti A, et al. Caustic ingestion and oesophageal cancer: intra- and peri-tumoral fibrosis is associated with a better prognosis. *Eur J Cardiothorac Surg*. 2010;38:659-664.
26. Tian F, Zang WD, Hou WH, et al. Nuclear factor-κB signaling pathway constitutively activated in esophageal squamous cell carcinoma cell lines and inhibition of growth of cells by small interfering RNA. *Acta Biochim Biophys Sin*. 2006;38:318-326.
27. Hatata T, Higaki K, Tatebe S, et al. Immunohistochemical study of nuclear factor-kB expression in esophageal squamous cell carcinoma: prognostic significance and sensitivity to treatment with 5-FU. *Dis Esophagus*. 2012;25:716-722.
28. Bakhle YS, Botting RM. Cyclooxygenase-2 and its regulation in inflammation. *Mediat Inflamm*. 1996;5:305-323.
29. Shirvani VN, Ouatu-Lascar R, Kaur BS, et al. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: ex vivo induction by bile salts and acid exposure. *Gastroenterology*. 2000;118:487-496.
30. Yang GZ, Li L, Ding HY, et al. Cyclo-oxygenase-2 is overexpressed in Chinese esophageal squamous cell carcinoma, and correlated with NF-κB: an immunohistochemical study. *Exp Mol Pathol*. 2005;79:214-218.
31. Souza RH, Shewmake K, Beer DG, et al. Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. *Cancer Res*. 2000;60:5767-5772.
32. Liu X, Li P, Zhang ST, et al. COX-2 mRNA expression in esophageal squamous cell carcinoma (ESCC) and effect by NSAID. *Dis Esophagus*. 2008;21:9-14.
33. Liu JF, Jamieson GG, Wu TC, et al. A preliminary study on the postoperative survival of patients given aspirin after resection for squamous cell carcinoma of the esophagus or adenocarcinoma of the cardia. *Ann Surg Oncol*. 2009;16:1397-1402.
34. Mantraradi RVP, Fineberg N, Ansari R, et al. A phase II study of cisplatin, 5 fluorouracil (5-FU), radiation (RT) and celecoxib in patients with resectable esophageal cancer (EC): updated results from the Hoosier Oncology Group (HOG) study. *J Clin Oncol*. 2004;22:4052.
35. Kishimoto T. IL-6: from its discovery to clinical applications. *Int Immunol*. 2010;22:347-352.
36. Murray PJ. The JAK-STAT signaling pathway: input and output integration. *J Immunol*. 2007;178:2623-2629.
37. Ogata A, Chauhan D, Teoh G, et al. IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *J Immunol*. 1997;159:2212-2221.
38. Dvorakova K, Payne CM, Ramsey L, et al. Increased expression and secretion of interleukin-6 in patients with Barrett's esophagus. *Clin Cancer Res*. 2004;10:2020-2028.
39. Quante M, Bhagat G, Abrams J, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett's-like metaplasia. *Cancer Cell*. 2012;21:36-51.
40. Lukaszewicz-Zajac M, Mroczko B, Kozlowski M, et al. Higher importance of interleukin 6 than classic tumor markers (carcinoembryonic antigen and squamous cell cancer antigen) in the diagnosis of esophageal cancer patients. *Dis Esophagus*. 2012;25:242-249.
41. Hanahan D, Weinburg RA. The hallmarks of cancer. *Cell*. 2000;100:57-70.
42. Huang Y, Boynton RF, Blount PL, et al. Loss of heterozygosity involves multiple tumor suppressor genes in human esophageal cancers. *Cancer Res*. 1992;52:6525-6530.
43. Mathew R, Arora S, Khanna R, et al. Alterations in p53 and pRb pathways and their prognostic significance in oesophageal cancer. *Eur J Cancer*. 2002;38:832-841.
44. Bani-Hani K, Martin IG, Hardie LJ, et al. Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma. *J Natl Cancer Inst*. 2000;92:1316-1321.
45. Hoffmann D, Hoffmann I, El-Bayoumy K. The less harmful cigarette: a controversial issue. A tribute to Ernst L. Wynder. *Chem Res Toxicol*. 2001;14:767-790.
46. Freedman ND, Abnet CC, Leitzmann MF, et al. A prospective study of tobacco, alcohol, and the risk of esophageal and gastric cancer subtypes. *Am J Epidemiol*. 2007;165:1424-1433.
47. Pfeifer GP, Denissenko MF, Olivier M, et al. Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene*. 2002;21:7435-7451.
48. Reed E. Platinum-DNA adduct, nucleotide excision repair and platinum based anti-cancer chemotherapy. *Cancer Treat Rev*. 1998;24:331-344.
49. Livneh Z. DNA damage control by novel DNA polymerases: translesion replication and mutagenesis. *J Biol Chem*. 2001;276:25639-25642.

50. Zambon P, Talamini R, La Vecchia C, et al. Smoking, type of alcoholic beverage and squamous-cell oesophageal cancer in northern Italy. *Int J Cancer*. 2000;86:144-149.
51. Lee CH, Wu DC, Lee JM, et al. Carcinogenetic impact of alcohol intake on squamous cell carcinoma risk of the oesophagus in relation to tobacco smoking. *Eur J Cancer*. 2007;43:1188-1199.
52. Salaspuro M. Acetaldehyde: a cumulative carcinogen in humans. *Addiction*. 2009;104:551-553.
53. Hiyama T, Yoshihara M, Tanaka S, et al. Genetic polymorphisms and esophageal cancer risk. *Int J Cancer*. 2007;121:1643-1658.
54. Väkeväinen S, Tillonen J, Agarwal DP, et al. High salivary acetaldehyde after a moderate dose of alcohol in ALDH2-deficient subjects: strong evidence for the local carcinogenic action of acetaldehyde. *Alcohol Clin Exp Res*. 2000;24:873-877.
55. Yang SJ, Wang HY, Li XQ, et al. Genetic polymorphisms of ADH2 and ALDH2 association with esophageal cancer risk in southwest China. *World J Gastroenterol*. 2007;13:5760-5764.
56. Yokoyama A, Muramatsu T, Ohmori T, et al. Alcohol and aldehyde dehydrogenase gene polymorphisms and oropharyngolaryngeal, esophageal and stomach cancers in Japanese alcoholics. *Carcinogenesis*. 2001;22:433-439.
57. Riboli E, Norat T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *Am J Clin Nutr*. 2003;78:559-569.
58. Freedman ND, Park Y, Subar AF, et al. Fruit and vegetable intake and esophageal cancer in a large prospective cohort study. *Int J Cancer*. 2007;121:2753-2760.
59. Yokokawa Y, Ohta S, Hou J, et al. Ecological study on the risks of esophageal cancer in Ci-Xian, China: the importance of nutritional status and the use of well water. *Int J Cancer*. 1999;83:620-624.
60. Jakszyn P, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. *World J Gastroenterol*. 2006;12:4296-4303.
61. Hotchkiss JH. Preformed N-nitroso compounds in foods and beverages. *Cancer Surv*. 1989;8:295-321.
62. Ghavamzadeh A, Moussavi A, Jahani M, et al. Esophageal cancer in Iran. *Semin Oncol*. 200;28:153-157.
63. De Jong UW, Day NE, Mounier-Kuhn PL, et al. The relationship between the ingestion of hot coffee and intraesophageal temperature. *Gut*. 1972;13:24-30.
64. Ghadirian P. Thermal irritation and esophageal cancer in northern Iran. *Cancer*. 1987;60:1909-1914.

Jill E. Larsen, Ramaswamy Govindan, and John D. Minna

32

Molecular Basis of Lung Cancer

Lung cancer is the leading cause of cancer-related death in men and women in the United States, accounting for approximately 28% of total cancer deaths in 2012 despite comprising only about 14% of new cancer cases.[1] Decades of research have contributed to our understanding that lung cancer is a multistep process involving genetic and epigenetic alterations where resulting DNA damage transforms normal lung epithelial cells into lung cancer. It is not known whether all lung epithelial cells or only a subset of these cells (such as pulmonary epithelial stem cells or their immediate progenitors) are susceptible to full malignant transformation. In addition, although the tumor-initiating cell may have only a handful of mutations, as the tumor expands, cells may acquire additional mutations.[2] Smoking damages the entire respiratory epithelium, and thus "field cancerization" or "field defects" (molecular changes) are observed in histologically normal lung epithelium, as well as a variety of histologic preneoplastic/premalignant lesions, which also harbor molecular abnormalities common to the adjacent tumor.[3] The culmination of these changes leads to lung cancers exhibiting all the hallmarks of cancer, including self-sufficiency of growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.[4]

Lung cancer is a heterogeneous disease clinically, biologically, histologically, and molecularly. Understanding the molecular causes of this heterogeneity and determining how the molecular changes relate to the biologic behavior of lung cancer and their utility as diagnostic and therapeutic targets are important basic and translational research issues. The past 10 years has witnessed a revolution in genomic technologies to characterize genetic and epigenetic changes throughout the lung cancer genome. These include the recent application of "next-generation" ("NexGen") sequencing, which has led to genome-wide mutational analyses of lung cancers. Within the next several years there will be data on perhaps 1000 lung cancers, providing an unprecedented amount of information. The central issues will be to determine which of these mutations are "actionable"—that is, provide a guide for targeting therapy; which are "passenger" and which are "driver" mutations; how frequent the mutations are; how the mutations are related to other molecular changes (e.g., methylation and miRNA profiles); and which mutations provide information to identify important subgroups ("molecular portraits") of lung cancer that provide prognostic (survival information independent of therapy) and/or predictive (survival information dependent on the administration of specific therapies) utility. Identifying "acquired vulnerabilities" in lung cancer that can be therapeutically targeted is key. As a lung epithelial cell acquires oncogenic changes (such as a KRAS mutation), it must acquire other changes to allow the cell to tolerate the oncogenic changes. These acquired vulnerabilities are not present in normal tissue and thus are "synthetically lethal" with the oncogenic changes in tumors. Many of these are likely to be considered "passenger" mutations. Nevertheless, they may represent actionable targets as well as enrollment biomarkers for selecting patients.

National Cancer Institute Lung Cancer Specialized Program of Research Excellence (SPORE) (P50CA70907), N R (NNJ05HD36G). JEL supported by NHMRC Biomedical Fellowship (494511).

Molecular Epidemiology and Etiology

The two main types of lung cancer, non–small-cell lung cancer (NSCLC) (representing 80% to 85% of cases) and small-cell lung cancer (SCLC) (representing 15% to 20%) are identified based on histological, clinical, and neuroendocrine characteristics. NSCLC can be further histologically subdivided into adenocarcinoma, squamous carcinoma, large-cell carcinoma (including large-cell neuroendocrine lung cancers), bronchioloalveolar lung cancer, and mixed histologic types (e.g., adenosquamous carcinoma).

Although about 85% of lung cancers are caused by carcinogens present in tobacco smoke, 15% to 25% of lung cancer cases occur in lifetime "never smokers" (fewer than 100 cigarettes in a lifetime). Furthermore, more than 50% of newly diagnosed lung cancers in the United States occur in

Table 32-1 Molecular Differences between Smoking and Never Smoking Lung Cancers

Gene	Never Smoking	Smoking
TP53 mutations—overall	Less common	More common
TP53 mutations—G:C to T:A mutations	Less common	More common
KRAS mutations	Less common (0%-7%)	More common (30%-43%)
EGFR mutations	More common (45%)	Less common (7%)
STK11 mutations	Less common	More common
EML4-ALK fusions	More common	Less common
HER2 mutations	More common	Less common
Methylation index	Low	High
p16 methylation	Less common	More common
APC methylation	Less common	More common
Loss of hMSH2 expression	Common (40%)	Rare (10%)

Data summarized from the following reviews: References 21-23.

former smokers, in whom the damage caused by past smoking still led to the development of lung cancer. Although the general public associates lung cancer with smoking, the numbers of lung cancer cases occurring in lifetime never smokers and former smokers both present a huge public health problem. Thus, it will be important to identify non–smoking-related etiologies of lung cancer arising in never smokers, as well as methods to identify which former smokers are most likely to develop clinically evident lung cancer.

Lung Cancer in Never Smokers

Never-smoking lung cancers represent a disease that is epidemiologically, clinically, and molecularly distinct from smoking lung cancers. If considered independently, never-smoking lung cancers are the seventh most common cause of cancer death.[5] Never-smoking lung cancer occurs more frequently in women and East Asians, has a peak incidence at a younger age, targets the distal airways, is usually adenocarcinoma, and frequently is epidermal growth factor receptor (EGFR) mutant and therefore responsive to EGFR-targeted therapies. Table 32-1 outlines the molecular differences between smoking and never-smoking lung cancers.

Inherited Susceptibility to Lung Cancer

The study of inherited predisposition to lung cancer has been investigated. Multiple genome-wide association studies (GWASs) have associated single-nucleotide polymorphism (SNP) variations at 15q24-q25.1 (including genes encoding nicotinic acetylcholine receptor [nAChR] subunits [*CHRNA5*, *CHRNA3*, and *CHRNB4*]) with an increased risk of both nicotine dependence and developing lung cancer. Although meta-analyses have provided further evidence that variation at 15q25.1, 5p15.33, and 6p21.33 influences lung cancer risk, it is not yet known whether there is a mechanistic association of these polymorphisms and nicotine addiction, carcinogenic derivatives of nicotine exposure, or the effect of nicotine acting on nAChRs. In addition, a genome-wide linkage study of pedigrees containing multiple generations of lung cancer from the Genetic Epidemiology of Lung Cancer Consortium (GELCC) mapped a familial susceptibility locus to 6q23-25.[2] Regulator of G-protein signaling 17 (*RGS17*) is a putative causal gene within this locus where common variants were associated with familial but not sporadic lung cancer; however, it is likely that more than one genetic locus in the 6q region is influencing susceptibility.

Human Papilloma Virus–Mediated Lung Cancer

Human papilloma virus (HPV), an established human carcinogen (for both uterine cervical and head and neck cancer), has been proposed to play a role in lung cancer pathogenesis; however, published data remain controversial. The presence of HPV oncoproteins E6 and E7 leads to inactivation of tumor suppressors p53 and Rb, respectively. A meta-analysis of 100 publications comprising 7381 cases found that the incidence of HPV in lung cancer differed by geographical origin of the study (higher in China, Taiwan, other Asia, and South America and lower in Australia, Europe, and North America) and histological subtype, where HPV was slightly more common in squamous-cell carcinomas (SCCs). The detection of oncogenic variants of HPV in some tumors and our knowledge of HPV oncoproteins suggest that HPV infection will be a major etiologic feature in a subset of lung cancer. Given the differences in response of HPV-associated head and neck cancer to EGFR-targeted therapy, it will be important to characterize other molecular alterations in these lung cancers, and how they respond to various therapies.

Genomics: Tools for Identification, Prediction, and Prognosis

The molecular heterogeneity of lung cancer and utility in classifying lung tumors by the specific mutations driving

their growth is demonstrated in tumors harboring either the epidermal growth factor receptor *(EGFR)* tyrosine kinase (TK) mutations or the *EML4-ALK* fusion protein. These tumors exhibit exquisite sensitivity to EGFR TK inhibitors, such as gefitinib and erlotinib, or the ALK and ROS1 inhibitor crizotinib, respectively. Advances such as these have spurred considerable interest and excitement in the field of lung cancer to fully understand the complex genomic landscape.[6] It will now be possible to achieve this lofty goal with the use of massively parallel sequencing and the comprehensive cataloging of SNPs, structural variations, gene amplifications, deletions, methylation, messenger RNA (mRNA) expression, and alterations in microRNAs (miRNAs) and miRNA binding sites present in a genome. Initial sequencing studies of either a subset of cancer-related genes or single-lung tumors or cell lines found the lung cancer genome displayed high protein-altering mutation rates, perhaps indicative of the inherent heterogeneity found in lung tumors compared with tumors from other tissues. A comprehensive and systematic analysis of cancer genomes from large numbers of patients with lung cancer is critical to identify significant molecular alterations that drive the cancer phenotype and eventually to develop rational therapies.

Challenges: Sample Procurement and Informed Consent

Technical and ethical factors such as sample procurement and informed consent remain a significant challenge to the application of these technologies. Serial collection of tumor samples at various points during disease progression would contribute to our understanding of the clonal evolution of tumors and better define the molecular mechanisms underlying metastatic process and resistance to targeted therapy.

Transcriptome Profiling

Profiling the lung cancer transcriptome has imparted biologically and clinically relevant information such as novel dysregulated genes and pathways and gene signatures that can predict patient prognosis, response to treatment, and histology.[7] Predictive and prognostic mRNA profiling has real potential for refining the care of lung cancer patients, but progress has been limited. In an effort to overcome limitations of sample size and heterogeneity in previous studies, a multisite, blinded validation study of 442 lung adenocarcinomas examined whether the mRNA profile of primary tumors could robustly predict patient outcome either alone or in combination with clinicopathological factors.[8] This study developed several models (or signatures) which, for the most part, predicted outcome better than current clinical methods. However, critical review of published prognostic signatures in lung cancer found little evidence of any published signature being ready for clinical application, mostly because of limitations in study design and analysis. Expression of nuclear receptors (and later their co-regulators) in lung cancer may provide as good or better prognostic information than other mRNA expression signatures. Because nuclear receptors are also targets for therapeutic manipulation (via hormone agonists and antagonists), expression patterns in individual lung cancers may also provide insight for targeted therapy. Despite the complexities of mRNA profiling, the success of prognostic signatures in breast cancer suggests the importance of further research efforts.

Genome-Wide Copy Number Profiling

High-resolution mapping of copy number alterations in the lung cancer genome has identified single genes as targets of genomic gain or loss through improved definition of known aberrant regions or by the identification of focal alterations undetectable with earlier technology. The analysis of primary lung adenocarcinomas identified significant recurrent copy-number alterations, of which a majority were focal events, including some mutations previously unrecognized in lung cancer—for example, amplification at 14q13.3 targeting the transcription factor NKX2-1, which is discussed later. The ongoing work led by the National Cancer Institute's The Cancer Genome Atlas (TCGA) project has revealed regions of significant copy number alterations in lung SCCs. These include previously reported regions of copy number alteration containing SOX2, PDGFRA/KIT, EGFR, FGFR1/WHSC1L1, CCND1, and CDKN2A genes as well as novel findings, including amplifications containing NFE2L2, MYC, CDK6, MDM2, BCL2L1, and EYS and deletions of FOXP1, PTEN, and NF1.[9] Similar studies in NSCLC and SCLC cohorts have identified other novel drivers of lung carcinogenesis.

Genome-Wide Sequencing of Lung Cancers

TCGA plans to comprehensively characterize the genomic alterations in 1000 patients with NSCLC. Sequencing of squamous, adenocarcinoma, and SCLC has been completed, yielding a list of "significantly mutated genes" (SMGs) (Table 32-2). Analysis of 178 patients with SCC is complete and has revealed that lung SCC displays a bewildering array of genomic changes with a mean of 360 mutations in the exons

Table 32-2 Significantly Mutated Genes in Lung Cancer Subtypes Identified with Exome Sequencing

Adenocarcinoma	Squamous-Cell Carcinoma	SCLC
ARID1A	ANP32C	ADCY1
ATM	APC	BCLAF1
BRAF	BCL11A	C17orf108
BRD3	BCL2L1	CDYL
CBL	**BRAF**	CNTNAP2
CTNNB1	CDK6	COL22A1
EGFR	CDKN2A	COL4A2
FBXW7	CREBBP	DIP2C
FGFR3	CSMD1	ELAVL2
GOPC	DDR2	GRIK3
KEAP1	**EGFR**	GRM8
KIAA0427	EYS	KHSRP
KRAS	FAM123B	KIF21A
NF1	**FBXW7**	PLSCR4
PIK3CA	FGFR1	RASSF8
PPP2R1A	FOXP1	**RB1**
PTEN	HLA-A	RIMS2
RB1	HRAS	RUNX1T1
RBM10	**KEAP1**	SATB2
SETD2	MLL2	TMEM132D
SMAD4	MUC16	**TP53**
SMARCA4	**NF1**	ZDBF2
STK11	NFE2L2	
TP53	NOTCH1	
U2AF1	**PIK3CA**	
	PTEN	
	RB1	
	REL	
	SMAD4	
	SMARCA4	
	TNFAIP3	
	TP53	
	TSC1	
	VGLL4	
	WHSC1L1	
	WWOX	

Genes in **bold** are present in more than one histological subtype.
Data generated through of analysis of 183 lung adenocarcinomas[24]; TCGA project of 178 previously untreated, stage I-IV primary lung squamous cell carcinoma[9]; and 34 primary SCLC tumors and 17 SCLC cell lines.[25]

(including 228 nonsilent mutations), 165 genomic rearrangements, and 323 segments of copy number alterations per tumor.[9] Similar rates of genomic alterations have been reported from studies of genomic changes in lung adenocarcinoma, where the average mutation frequency in smokers with adenocarcinoma of the lung was 10-fold higher compared with lifelong never smokers. Substantial differences were also found not just in mutational burden but also in the mutational spectra of affected genes between the smokers and lifelong never smokers with lung adenocarcinoma. Ongoing analyses of lung adenocarcinoma and other lung cancer subtypes such as SCLC by TCGA and other groups (see Table 32-2) will better determine significant "actionable" genes.

Identification of Novel Pathways

TCGA project found a significant number of lung SCCs had alterations in genes involved in oxidative stress response and squamous differentiation. Genomic alterations included point mutations and copy number alterations. More specifically, alterations in one of the three genes *NFE2L2*, *KEAP1*, and *CUL3* were identified in nearly a third of tumor samples studied. The master antioxidant transcription factor NFE2L2 promotes survival following cellular insults that trigger oxidative damage and is regulated by KEAP1, an oxidative stress sensor. In unstressed conditions, KEAP1 binds and subsequently represses NFE2L2. KEAP1 also forms a ubiquitin E3 ligase complex with CUL3, resulting in constant ubiquitination of NFE2L2. Mutations in *NF2L2* occurred nearly exclusively in one of the two KEAP1 interaction motifs. Mutations in *KEAP1* and *CUL3* showed a pattern consistent with loss of function. In addition, mutations in *KEAP1* and *CUL2* were mutually exclusive with *NFE2L2*. Alterations in genes that are known to play a role in squamous differentiation were identified in 44% of lung SCC samples. The changes include overexpression and amplification of SOX2 and TP63 and loss-of-function mutations involving *NOTCH1* and *NOTCH2*. Truncating mutations involving *NOTCH1* and *NOTCH2* have been reported previously in squamous cell cancer of the skin and lung.

Recurrent somatic mutations in the splicing factor gene *U2AF1*, truncating mutations affecting *RBM10* and *ARID1A*, and in-frame exonic alterations within EGFR and SIK2 kinases were identified in an exome and genome analysis of lung adenocarcinoma. *SOX2* mutations and amplification and a recurrent *RFL-MYCL1* fusion were common in an exome, transcriptome, and copy number analysis of 34 primary SCLC tumors and 17 SCLC cell lines. Suppression of *SOX2* in *SOX2*-amplified cell lines or *MYCL1* in *RLF-MYCL1* cell lines both resulted in decreased proliferation, suggesting that these alterations may represent SCLC subtype vulnerabilities.

Identification of Therapeutic Targets

The lung SCC TCGA project reported a number of potentially targetable alterations using a gene-centered and pathway-directed approach. Using fairly stringent criteria (availability of a targeted agent, confirmation of altered allele in transcriptome sequencing, and Mutation Assessor Score), a potentially targetable gene was identified in 64% of samples studied. Alterations in one of the three core pathways (PI3K/AKT/mTOR, RTKs, and RAS/RAF/MAPK) were found in 69% of samples even after restricting the analysis to include only those where mutations were confirmed by transcriptome sequencing and those amplifications associated with overexpression of the target gene. Some of the notable targets altered include PI3KCA, PTEN, AKT3, BRAF, FGFR, and EGFR. Another novel target identified with whole-transcriptome analyses of tumor samples is in-frame fusion transcripts

involving *KIF5B* (the Kinesin family 5B gene) and the *RET* oncogene, which is found in 1% to 2% of patients with lung adenocarcinoma and is discussed later.

Lessons Learned and Future Directions

Preliminary TCGA analysis of lung SCCs has demonstrated the importance of integrating mutational data with other genomic data such as methylation, mRNA expression, and copy number. *CDKN2A*, a tumor suppressor gene (TSG) that encodes two cell cycle inhibitor proteins, p16 and p14, is frequently altered in lung SCC. *CDKN2A* is inactivated through multiple mechanisms from epigenetic silencing by methylation (21%), to inactivating mutation (18%), to other events such as exon skipping (4%) and homozygous deletion (29%). Thus considering only one set of genomic data could lead to inaccurate conclusions on the role of the gene.

It is clear that next-generation sequencing has enormous potential to unravel the complexities of the lung cancer genome and identify the molecular mechanisms underpinning therapeutic responses and progression of lung cancer. Although the challenges in gathering reliable and clinically and pathologically annotated data are not trivial, high-throughput technologies and publicly stored genome-wide databases related to lung cancer are resources with the potential to drive a global collaborative effort in identifying new targets for lung cancer diagnostics and therapeutics. Large-scale multidisciplinary and international collaborations such as the TCGA project, the NCI Lung Cancer Mutation Consortium (LCMC),[10] as well as international lung cancer sequencing consortiums will enable the uniting of clinically annotated with molecularly annotated lung cancer specimens. Enabling free access to all of these genome-wide studies will allow independent confirmation on the role of the various molecular changes for prognosis, prediction, and targeting of therapy of lung cancer.

Genome-Wide Functional (siRNA, shRNA Library) Screening

"Synthetic lethal" screens using RNAi (siRNAs and shRNA libraries) technology have allowed unbiased, genome-wide approaches to identification of genes whose perturbation can selectively kill lung cancer cells. The ability to identify "synthetic lethality"[11] associated with oncogenic changes in tumor cells has particular utility in identifying new therapeutic targets or molecules to treat traditionally hard-to-target tumors, such as those with oncogenic KRAS. Small interfering RNA (siRNA) and short-hairpin RNA (shRNA) screens have identified genes whose perturbation can selectively sensitize NSCLC cell lines to sublethal doses of chemotherapeutic agents, sensitize KRAS mutant cells to targeted drugs, suppress tumorigenicity in cells with specific gene dysregulation such as oncogenic KRAS or aberrant EGFR, and identify novel genes critical for tumorigenic processes such as metastasis.

Epigenetic Changes in Lung Carcinogenesis

Epigenetic events can lead to changes in gene expression without any changes in DNA sequence and therefore, importantly, are potentially reversible.

Methylation and Histone Modification

Aberrant promoter hypermethylation (the addition of a methyl group to CpG islands in the promoter region of a gene that results in transcriptional silencing) is a common method for inactivation of TSGs in tumor cells and occurs early in lung tumorigenesis. In fact, whole-genome microarray profiling of DNA methylation patterns in lung cancer—termed the *lung cancer epigenome* or *methylome*—suggests that the role of methylation in lung tumorigenesis may have been previously underestimated. Because aberrant methylation is an early event in lung cancer pathogenesis and is detectable in DNA circulating in the blood, many studies have investigated methylation status as a biomarker for risk assessment, early detection, disease progression, and prognosis in lung cancer (Table 32-3).

DNA is methylated by DNA methyltransferases (DNMTs) which are responsible for both de novo and maintenance of preexisting methylation in a cell. Histone modification is another mechanism for epigenetic control of gene transcription: histone deacetylation results in condensing of chromatin, resulting in transcriptionally inactive DNA. Inhibitors of DNMTs or histone deacetylases (HDACs) resulting in pharmacologic restoration of expression of epigenetically silenced genes is an exciting targeted therapeutic approach and shows promise in lung cancer (Table 32-4).

microRNA-Mediated Regulation

There is currently a strong research focus on microRNAs (miRNAs) as potential diagnostic and prognostic biomarkers and therapeutic targets for lung cancer. miRNA profiles for histologic and prognostic classification of lung tumors and detection of miRNAs in peripheral blood and sputum illustrate the potential of miRNAs as diagnostic and early

Table 32-3 DNA Methylation as a Biomarker in Lung Cancer

Early Detection	Prognostic	Predictive
APC	APC	SFN (14-3-3 sigma)
CDH13	CDH1	
DAPK1	CDH13	
DNMT1	CXCL12	
FHIT	DAPK1	
GATA5	DLEC1	
GSTP1	EPB41L3 (DAL-1)	
MAGEA1	ESR1	
MAGEB2	FHIT	
MGMT	IGFBP-3	
p16	MGMT	
PAX5-b	MLH1	
RARβ2	MSH2	
RASSF1A	p16	
RASSF5	PYCARD (ASC)	
RUNX3	PTEN	
TCF21	RASSF1A	
	RRAD	
	RUNX3	
	SPARC	
	TIMP3	
	TMS1	
	TSLC1	
	WIF1	

Data summarized from the following reviews: References 26-28.

detection biomarkers in lung cancer. Table 32-5 summarizes some experimentally validated miRNAs important in lung cancer. miRNAs are a class of non–protein-encoding small RNAs capable of regulating gene expression by either directly cleaving a targeted mRNA or inhibiting translation by interacting with the 3′ untranslated region (UTR) of a target mRNA. A single miRNA often targets multiple genes, and multiple miRNAs may target the same mRNA, which results in a complex network of molecular pathways where a single miRNA (to date, more than 1400 human miRNAs have been identified) can potentially affect multiple cellular processes. Aberrant expression of miRNAs has been found to play an important role in the pathogenesis of lung cancer as either oncogenes or TSGs. miRNAs can function as either TSGs or oncogenes. Restoration of aberrantly expressed miRNAs can be achieved in vitro and in vivo using miRNA mimics (for underexpressed miRNAs) or miRNA inhibitors (termed *antisense oligonucleotides* or *antagomirs;* for overexpressed miRNAs). Concurrent inhibition or overexpression of miRNAs with conventional therapies has resulted in an

Table 32-4 Targeted Therapies Approved in Clinical Trial or in Preclinical Study for Lung Cancer

Pathway	Target	Clinically Approved for Lung Cancer	In Clinical Trials for Lung Cancer	Novel Agents in Preclinical Study
RTKs	EGFR	Erlotinib, cetuximab*	Afatinib, BMS-690514, canertinib, CUDC-101, EKB-569, gefitinib,† icotinib, lapatinib, matuzumab, necitumumab, neratinib, nimotuzumab, panitumumab, pelitinib, PF0299804, vandetanib, XL647, zalutumumab	AEE 788, AV-412, BMS-599626
	VEGFR		Axitinib, BMS-690514, brivanib alaninate, cediranib, E7080, foretinib, linifanib, motesanib, neovastat, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, tivozanib, vandetanib, vargatef, vatalanib, XL184, XL647, XL999	Adnectin, AEE 788, TKI-258, TSU-68
	ALK	Crizotinib		GSK1838705A, nVP-TAE684
	HER2		Afatinib, BMS-690514, CI-1033, CUDC-101, EKB-569, lapatinib, PF0299804, neratinib, pertuzumab, trastuzumab, XL647	AEE 788, AV-412, BMS-599626
	c-MET	Crizotinib	AMG 102, AV-299, foretinib, GSK1363089, MetMAb, tivantinib, XL184	AMG 208, PF-04217903, PHA-665752, SCH900105, SGX523, SU11274
	PDGFR		Axitinib, cediranib, dasatinib, E7080, imatinib, IMC-3G3, linifanib, motesanib, pazopanib, ramucirumab, regorafenib, sorafenib, sunitinib, vargatef, vatalanib, XL999	TKI-258, TSU-68
	IGF-1R		AMG 479, BIIB022, cixutumumab, figitumumab, MK-0646, OSI906	BMS-754807
	FGFR		Brivanib alaninate, E-7080, regorafenib, TKI-258, vargatef, XL999	FP-1039, PD-173074, TSU-68
	c-KIT		Axitinib, cediranib, dasatinib, imatinib, motesanib, pazopanib, regorafenib, sorafenib, sunitinib, vatalanib	
	FLT-3		MK-0457, sorafenib, sunitinib, XL999	
	SRC/BCR-ABL		AZD0530, dasatinib, imatinib, XL999	KX2-391
	DDR2		Dasatinib	
Angiogenesis	VEGF	Bevacizumab	Aflibercept, AMG706, cediranib	
RAS/RAF/MAPK	RAS		Tipifarnib	Lonafarnib, ISIS 2503 (H-Ras)
	RAF		GSK2118436, regorafenib, sorafenib	AZ628, ISIS 5132, XL281
	MEK		GSK1120212, PD325901, selumetinib, sorafenib	AS 703026, AZD8330, GDC-0973, RDEA119
PI3K/AKT/mTOR	PI3K		BKM120, GDC-0941, PX-866, XL147, XL765	BEZ235, BGT226, LY294002
	AKT		Nelfinavir, MK-2206, perifosine	
	mTOR		Everolimus, PX-866, ridaforolimus, sirolimus/rapamycin, temsirolimus	AZD 8055, BEZ235, OSI-027,

Table 32-4 Targeted Therapies Approved in Clinical Trial or in Preclinical Study for Lung Cancer—cont'd

Pathway	Target	Clinically Approved for Lung Cancer	In Clinical Trials for Lung Cancer	Novel Agents in Preclinical Study
Apoptosis	IAPs TRAIL BCL2 PARP FUS1		 Conatumumab, dulanermin, mapatumumab Gossypol, navitoclax, oblimersen, obatoclax Iniparib, veliparib	HGS01029 Apomab, lexatumumab ABT-737 AG014699, olaparib fus1 liposome complex
Heat shock proteins	HSP90		Ganetespib, retaspimycin, SNX-5422, tanespimycin	17-AAG, alvespimycin
HDACs	HDACs		Belinostat, CI-994, CUDC-101, entinostat, panobinostat, pivanex, romidepsin, vorinostat	SB939
Proteasome	Proteasome		Carfilzomib, bortezomib, salinosporamide A	CEP-18770, MLN9708,
Stem cell pathways	Hh (SMO) Notch (γ-secretase)		RO4929097, vismodegib, XL139	Cyclopamine, IPI-926, LDE225 MK0752, MRK-003, PF03084014
Telomerase	Telomerase		Imetelstat, KML-001	Sodium meta arsenite
Cell cycle/cell proliferation	p53 MDM2 Aurora kinase CDK			p53 peptide vaccine, PRIMA-1 JNJ-26854165, RO5045337 AZD 1152, alisertib, MK0457, MK5108 Purvalanol
Inflammation	COX-2 TGF-β PPARγ		Celecoxib Iniparib, CS 7017	 Trabedersen Olaparib
Hypoxia	HIF1		PX-478	

*Although not currently U.S. FDA-approved for non–small-cell lung cancer, cetuximab is a recommended treatment in several practice guidelines, including those of the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN).
†Previously approved in the United States and still approved elsewhere.
Adapted from Reference 29.

Table 32-5 miRNAs with Diagnostic, Prognostic, and/or Predictive Roles in Lung Cancer

miRNA	Expression in Lung Cancer	Correlation with Poor Prognosis* and/or Predictive Role	Validated Targets
Tumor-Promoting miRNAs			
miR-17-92 cluster	Up		PTEN , E2F1-3, BIM
miR-21	Up	Positive; resistance to EGFR targeted therapy	PTEN, SPRY1, PDCD4, RALGDS
miR-155	Up†	Positive	CK1α, TP53INP1, MMR
miR-221/222	Up	Positive; resistance to TRAIL treatment	PTEN, TIMP3, cKIT, p27Kip1
Tumor-Suppressing miRNAs			
let-7/miR-98	Down	Negative	RAS, MYC, HMGA2, CDC25A, CDK6, CCND1
miR-15/16	Down		BCL1, MCL1, CCND1, WNT3A
miR-29	Down		DNMT3A-3B, MCL1
miR-34a-c	Down	Negative	SIRT1, BCL2, CD44, CDK4/6, CCNE2, MYC, E2F3
miR-128b	Down	Negative (gefitinib treated)	EGFR
miR-200 family	Down		ZEB1/ZEB2

Data summarized from the following reviews: References 31-34.
*Correlation with poor prognosis.
†In KRAS and EGFR wild-type tumors.

increased response to EGFR TKIs, radiotherapy, and chemotherapy. These studies illustrate the potential of miRNAs in lung cancer therapeutics development; however, limitations in pharmacokinetics, delivery, and toxicity need to be addressed.

The *let-7* family is a cluster of miRNAs that function as tumor suppressors and is frequently underexpressed in lung tumors, particularly NSCLC, compared to normal lung, and decreased expression has been associated with poorer prognosis. *Let-7* regulates multiple oncogenes including RAS, MYC, and HMGA2 via binding to the let-7 binding sites in their respective 3′ UTRs. *Let-7* replacement therapy shows potential, with reduced tumor burden observed in vivo; however, tumor response in patients will be affected by a SNP in the *let-7* complementary site (LCS6) of KRAS, which is significantly associated with lung cancer risk and results in increased KRAS expression in vitro.

An example of an important oncogenic miRNA—oncomir—in lung cancer is RAS-regulated *miR-21* which promotes cellular growth and invasion and metastasis by targeting multiple genes with tumor suppressive effects such as negative regulators of the RAS/RAF/MAPK pathway, pro-apoptotic, and anti-metastatic genes. Expression of *miR-21* is also suggested to be positively regulated by the EGFR signaling pathway, specifically EGFR mutations. Some miRNAs have also been shown to be important mediators of metastasis. The expression of miR-200 family members is commonly lost in aggressive lung cancers and can prevent epithelial to mesenchymal transition (EMT)—and consequently, invasion and metastasis—by repressing transcriptional repressors of E-cadherin.

Oncogenes, Tumor Suppressor Genes, and Signaling Pathways in Lung Cancer

The "hallmarks of cancer"[4] describe the complexities of neoplastic disease and stratify the complexities by mechanistic function. Genomic instability is an underlying "enabling" characteristic of lung cancer cells where alterations such as chromosomal rearrangements can generate rare genetic events in cells that eventually give rise to cancer. Mapping amplifications and deletions in copy number throughout the cancer genome has led to the identification of many oncogenes and TSGs. Recent whole-genome genomic approaches have yielded further insight into the complexities of the lung cancer genome with the identification of driving mutations and other key signaling pathways (see Table 32-2). The following section summarizes known driver mutations (*EGFR*, *KRAS*, and EML4-ALK) and key signaling pathways (including RAS/RAF/MAPK, PI3K/AKT/mTOR, p53, and p16/RB) in lung cancer organized by "hallmarks" (Figure 32-1).[12] There are several targeted therapy agents in the clinic or in development for lung cancer (see Table 32-4).

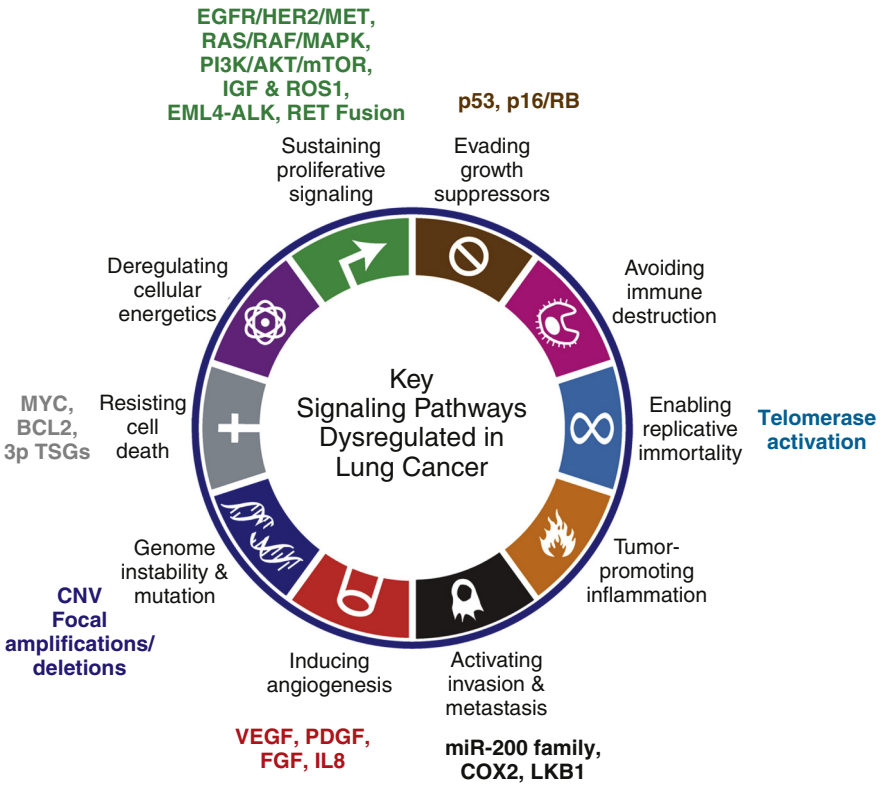


Figure 32-1 Key signaling pathways discussed in this chapter that are commonly dysregulated in lung cancer in relation to the "Hallmarks of Cancer" proposed by Hanahan and Weinberg[4] Currently, most of our knowledge of the molecular changes in lung cancer converges on the six original hallmarks (sustaining proliferative signaling; evading growth suppressors; resisting cell death; enabling replicative immortality; inducing angiogenesis; and activating invasion and metastasis), as well as the newly categorized "enabling characteristic" genome instability and mutation. *(Reprinted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation.* Cell. *2011;144:646-674, with permission from Elsevier.)*

Oncogene activation probably occurs in all lung cancers (typically by gene amplification, overexpression, point mutation, or DNA rearrangements), resulting in persistent upregulation of mitogenic growth signals, which induce cell growth. Importantly, it can also result in "oncogene addiction" in which the cell becomes dependent on this aberrant oncogenic signaling for survival.[13] These "driver" oncogenes or oncogene "addictions" represent acquired vulnerabilities in lung cancer cells and present as significant therapeutic targets by offering the specificity of killing tumor but not normal cells.

Loss of TSG function is an important step in lung carcinogenesis and usually results from inactivation of both alleles, with loss of heterozygosity (LOH; through chromosomal deletion or translocation) inactivating one allele, and point mutation or epigenetic or transcriptional silencing inactivating the second allele. Commonly inactivated TSGs in lung cancer include *TP53*, *RB1*, *STK11*, *CDKN2A*, *FHIT*, *RASSF1A*, and *PTEN*. Historically, tumor suppressors have been more difficult to target with therapeutic agents because restoration of lost activity is much more difficult than inhibition of increased activity (as with oncogenes), and consequently most endeavors were targeted at downstream effectors. Increased understanding of the function of tumor suppressor proteins may identify novel therapeutic targets, as shown with p53, where compounds that stabilize the mutant protein or restore wild-type conformation demonstrate clinical utility.

Hallmark: Sustaining Proliferative Signaling

EGFR/HER2/MET Signaling

EGFR

The ErbB family of tyrosine kinase receptors includes four members: EGFR, ERBB2 (HER2), ERBB3, and ERBB4. The receptors can activate multiple signal transduction cascades, including the RAS/RAF/MAPK, PI3K/AKT/mTOR, and STAT pathways, by forming homo- and heterodimers with different ligand specificity. EGFR is overexpressed or aberrantly activated in 50% to 90% of NSCLCs. EGFR-targeted inhibitors include monoclonal antibodies that target the EGFR extracellular domain and tyrosine kinase inhibitors (TKIs), which are small molecules that inhibit intracellular tyrosine kinase activity of EGFR. A significant advance was made in the treatment of NSCLC with the observation that lung cancer somatic mutations in the kinase domain of EGFR strongly correlated with sensitivity to EGFR TKIs. EGFR mutant lung tumors exhibit exquisite sensitivity and marked tumor response with EGFR TKIs (such as erlotinib and gefitinib) and antibodies (such as cetuximab)—an example of oncogene addiction in lung cancer where tumors driven by EGFR mutation-activation of EGF signaling rely on continued EGF signaling for survival. "Classic" EGFR mutations in the tyrosine kinase domain (by either exon 19 deletion or exon 21 L858R mutation, which each account for about 45% of EGFR mutations) show an increased amount and duration of EGFR activation compared with wild-type receptors and have preferential activation of the PI3K/AKT/mTOR and STAT3/STAT5 pathways rather than the RAS/RAF/MAPK pathway. By contrast, the remaining 10% of EGFR tyrosine kinase mutations, in exons 18 and 20, do not confer sensitivity to EGFR TKIs and in some cases are associated with EGFR TKI resistance. EGFR mutations of all types are particularly prevalent in certain patient subgroups: adenocarcinoma histology, women, never smokers, and East Asian ethnicity. Despite an initial response, patients treated with EGFR TKIs eventually develop resistance to TKIs that is linked (in approximately 50% of tumors) to a T790M mutation in EGFR exon 20. In such cases, it is likely that a small population of cancer cells harboring T790M mutations is present at diagnosis and selected for during EGFR TKI therapy. Proposed mechanisms of the T790M-associated therapeutic resistance include a conformational change resulting in steric hindrance to EGFR TKI binding and increased EGFR affinity for ATP. Resistance to TKI therapy has also been associated with EGFR exon 20 insertions; KRAS mutation; amplification or activation of the *MET* proto-oncogene, which provides an alternative signaling pathway; and occasionally a switch of tumor differentiation to an SCLC-like phenotype. Second-generation EGFR TKIs (such as PF00299804, afatinib, and neratinib) bind irreversibly to EGFR tyrosine kinase, induce much less therapeutic resistance, and appear effective against secondary resistance mutations such as T790M.

ERBB2 (HER2)

The ligand for HER2 remains unknown, but HER2 is activated following homo- or heterodimerization (with EGFR or HER3 preferentially). Unlike breast and gastric cancers, HER2 amplification or overexpression in NSCLC does not confer sensitivity to HER2 antibodies or TKIs. However, exon 20 mutations in HER2 mutations (occurring in 3% to 10% of lung adenocarcinomas) do confer sensitivity to lapatinib in NSCLC cell lines. HER2 mutations also confer resistance to EGFR TKIs regardless of EGFR mutation status as HER2 replaces EGFR in driving growth signals.

MET

Similar to EGFR, MET is a receptor tyrosine kinase capable of driving RAS/RAF/MAPK and PI3K/AKT/mTOR pathway signaling following activation on hepatocyte growth factor (HGF) binding. Amplification of *MET* is also thought to mediate resistance to EGFR TKIs, independent of the

T790M mutation, where MET activates the PI3K/AKT/mTOR pathway through phosphorylation of HER3, independent of EGFR and HER2. Inhibition of MET is being successfully approached with antibodies (such as MetMAb) and small-molecule MET inhibitors (tivantinib/ARQ-197) (see Table 32-4).

RAS/RAF/MAPK Pathway

The RAS proto-oncogene family (KRAS, HRAS, NRAS, and RRAS) encodes four highly homologous 21-kDa membrane-bound proteins involved in signal transduction. Activation of the RAS/RAF/MAPK pathway occurs frequently in lung cancer, most commonly via activating mutations in KRAS (approximately 20%, particularly adenocarcinomas). In lung cancer, 90% of mutations are located in *KRAS* (80% in codon 12, and the remainder in codons 13 and 61), with *HRAS* and *NRAS* mutations only occasionally documented. Proteins encoded by the *RAS* genes exist in two states: an active state, in which GTP is bound to the molecule, and an inactive state, where the GTP has been cleaved to GDP. Activating point mutations confer oncogenic potential through loss of intrinsic GTPase activity, resulting in an inability to cleave GTP to GDP. This results in constitutive activation of downstream signaling pathways, such as PI3K and MAPK, rendering KRAS mutant tumors independent of EGFR signaling and therefore resistant to EGFR TKIs as well as chemotherapy. KRAS mutations are mutually exclusive with EGFR and ERBB2 mutations and are primarily observed in lung adenocarcinomas of smokers. The prevalence and importance of KRAS in lung tumorigenesis make it an attractive therapeutic target. Two unsuccessful approaches were farnesyltransferase inhibitors, to inhibit posttranslational processing and membrane localization of RAS proteins, and antisense oligonucleotides against RAS. More recently, efforts have been centered on downstream effectors of RAS signaling: RAF kinase and mitogen-activated protein kinase (MAPK) kinase (MEK).

BRAF is the direct effector of RAS. Although it is commonly mutated in melanoma (about 70%), mutations are rare in lung cancer (about 3%), predominantly in adenocarcinoma, and mutually exclusive to EGFR and KRAS mutations. Strategies to inhibit RAF kinase include degradation of *RAF1* mRNA through antisense oligodeoxyribonucleotides, and inhibition of kinase activity with small molecule kinase inhibitors such as the multikinase inhibitor sorafenib (which inhibits VEGFR, PDGFR, FLT-3, RAF, MEK, and KIT) as well as some BRAF mutant-specific inhibitors such as vemurafenib, PLX-4720, and GDC-0879. Several potent and selective MEK inhibitors such as selumetinib (AZD6244) and GSK1120212 show potential in inhibiting RAS/RAF/MAPK signaling (see Table 32-4). Attempts to directly inhibit or perturb mutant KRAS continue with the advent of whole-genome approaches. Synthetic lethal siRNA screens have identified siRNAs that specifically kill human lung cancer cells with KRAS mutations in vitro. In addition, the combination of anti-KRAS strategies (such as depletion with shRNAs) with other targeted drugs has shown potential therapeutic utility.

PI3K/AKT/mTOR Pathway

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that regulate cellular processes such as proliferation, survival, adhesion, and motility. The PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is downstream of several receptor tyrosine kinases including EGFR, and downstream effectors are involved in cell growth, angiogenesis, cell metabolism, protein synthesis, and suppression of apoptosis directly or via the activation of mTOR. In lung tumorigenesis, activation of the PI3K/AKT/mTOR pathway occurs early in pathogenesis, generally through mutations or amplification of (oncogenes) PI3K (as well as EGFR or KRAS), activation of AKT, or PTEN loss of function (TSG), and promotes cell survival through inhibition of apoptosis. PTEN, TSC1, TSC2, and STK11 (LKB1) are tumor suppressors that function as negative regulators of the pathway, and thus their loss of function activates the pathway. PTEN antagonizes the PI3K/AKT/mTOR pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3), a product of PI3K, to PIP2 and is commonly inactivated in lung cancer by mutations or loss of expression. TSC1 and TSC2 form a complex that inhibits activity of small G protein Rheb, leading to inhibition of the mTOR complex mTORC1. TSC1/TSC2-mediated inhibition of mTORC1 can be activated by LKB1 and AMPK and inhibited by AKT-mediated phosphorylation of TSC2. The serine/threonine kinase mTOR, a downstream effector of AKT, is an important intracellular signaling enzyme in the regulation of cell growth, motility, and survival in tumor cells. Molecular characterization of PI3K/AKT/mTOR pathway biomarkers (such as loss of PTEN) will enable better selection of tumors responsive to mTOR, AKT, and PI3K inhibition.

STK11 (LKB1)

The serine/threonine kinase STK11 (also called LKB1) functions as a TSG by regulating cell polarity, motility, differentiation, metastasis, and cell metabolism. Germline inactivating mutations of STK11 cause Peutz-Jeghers syndrome, but somatic inactivation through point mutation and frequent deletion on 19p13 occurs in approximately 30% of lung cancers—making it the third most commonly mutated gene in lung adenocarcinoma after p53 and RAS. STK11 mutations often correlate with KRAS activation and result in the promotion of cell growth. Its tumor-suppressing

effect is thought to function, in part, through inhibition of the mTOR pathway via AMP-activated protein kinase. STK11 inactivation appears to be particularly prevalent in NSCLC but rare in SCLCs; inactivating mutations are more common in tumors from males and smokers and in poorly differentiated adenocarcinomas. Mutation in both *KRAS* and *STK11* appears to confer increased sensitivity to MEK inhibition in NSCLC cell lines compared to either mutation alone.

Insulin Growth Factor (IGF) Pathway and ROS1

The insulin growth factor (IGF) pathway mediates the growth and differentiation of bone and skeletal muscle and comprises two receptors (insulin receptor [IR] and insulin-like growth factor-1 receptor [IGF-1R]) and three principal ligands (IGF-1, IGF-2, and insulin). IGF-1R is a receptor tyrosine kinase that forms homo- and heterodimers with IR and HER2. Activation on ligand binding results in upregulation of various signaling pathways including the PI3K/AKT/mTOR and RAS/RAK/MAPK pathways. Dysregulation of IGF signaling in lung cancer is evidenced by frequent (up to 70%) overexpression of IGF-1R in NSCLC, where increased signaling results in tumor growth and drug resistance. Furthermore, increased plasma levels of IGF-1 are associated with increased risk of lung cancer.

ROS1 is a receptor tyrosine kinase in the insulin receptor family. Rearrangements involving the *ROS1* gene were initially described in glioblastoma but have now been reported in lung cancer and other malignancies. *ROS1* rearrangement, as determined by FISH, has been reported in 1% to 2% of patients with NSCLC. Patients whose tumor cells exhibit *ROS1* rearrangement tend to be younger (median age around 50 years) and lifelong nonsmokers. Crizotinib appears to have promising activity in this molecular subset of NSCLC.

Other Fusion Proteins: EML4-ALK and RET

EML4-ALK

A novel fusion gene with transforming ability was reported in a small subset of NSCLC patients. Formed by the inversion of two closely located genes on chromosome 2p, fusion of PTK echinoderm microtubule-associated protein like-4 (EML4) with anaplastic lymphoma kinase (ALK), a transmembrane tyrosine kinase, yields the EML4-ALK fusion protein. The fusion results in constitutive oligomerization leading to persistent mitogenic signaling and malignant transformation. Meta-analysis of 13 studies encompassing 2835 tumors reported that the EML4-ALK fusion protein is present in 4% of NSCLCs. *EML4-ALK* fusions are, in nearly every case, found exclusive of *EGFR* and *KRAS* mutations and occur predominantly in adenocarcinomas, never or light smokers, younger patients, and males. Tumors with EML4-ALK fusions exhibit dramatic clinical responses to ALK targeted therapy, and the ALK and MET inhibitor crizotinib (PF-02341066) is now approved for use for lung cancer treatment in patients harboring the fusion protein.

RET

Whole-transcriptome analyses of tumor samples revealed the presence of in-frame fusion transcripts involving *KIF5B* (the Kinesin family 5B gene) and *RET* oncogene in 1% to 2% of patients with lung adenocarcinoma. In vitro studies have shown that *KIF5B-RET* is capable of inducing malignant transformation and its effect can be reversed with a *RET* kinase inhibitor. Although further studies are needed to evaluate the therapeutic role of *RET* fusions in lung cancer, this demonstrates how next-generation sequencing has opened a new field of investigation for therapeutic approaches.

Hallmark: Resisting Cell Death and Evading Growth Suppressors

MYC

One of the major downstream effectors of the RAS/RAF/MAPK pathway is the MYC proto-oncogene. In normal conditions, this transcription factor functions to keep tight control of cellular proliferation; however, aberrant expression through amplification or overexpression is commonly found in lung cancer. MYC proto-oncogene members (MYC, MYCN, and MYCL) are targets of RAS signaling and key regulators of numerous downstream pathways such as cell proliferation,[14] where enforced Myc expression drives the cell cycle in an autonomous fashion. It can also sensitize cells to apoptosis through activation of the mitochondrial apoptosis pathway—thus, Myc-driven tumorigenesis often requires coexpression of anti-apoptotic BCL2 proteins. Activation of MYC members often occurs through gene amplification, with MYC most frequently activated in NSCLC and all three members (MYC, MYCN, and MYCL) activated in SCLC by amplification. Recently, genome-wide analyses have identified MYCL translocations as a frequent mechanism of activation in SCLC.

The 3P Tumor Suppressor Genes: Regulators of Apoptosis

Loss of one copy of chromosome 3p is one of the most frequent and early events in human cancer, found in 96% of lung tumors and 78% of lung preneoplastic lesions. Mapping of this loss identified several candidate TSGs, including *FHIT* (3p14.2), *RASSF1A*, *TUSC2* (also called *FUS1*),

and semaphorin family members *SEMA3B* and *SEMA3F* (all at 3p21.3), and *RAR*β (3p24). In addition to LOH or allele loss, some 3p genes often exhibit decreased expression in lung cancer cells because of promoter hypermethylation. *FHIT*, located in the most common fragile site in the human genome (*FRA3B*), has been shown to induce apoptosis in lung cancer. *RASSF1A* can induce apoptosis, stabilize microtubules, and affect cell cycle regulation. *TUSC2* mediates apoptosis in cancer cells but not normal cells by upregulation of the intrinsic apoptotic pathway and inhibits several protein tyrosine kinases such as EGFR, PDGFR, c-Abl, c-Kit, and AKT. The candidate TSG *SEMA3B* encodes a secreted protein that can decrease cell proliferation and induce apoptosis when reexpressed in lung, breast, and ovarian cancer cells, in part by inhibiting the AKT pathway. Another family member, *SEMA3F*, may inhibit vascularization and tumorigenesis by acting on VEGF and ERK1/2 activation. *RAR*β exerts its tumor-suppressing function by binding retinoic acid, thereby limiting cell growth and differentiation.

The p53 Pathway

TP53 (17p13) encodes a phosphoprotein that prevents accumulation of genetic damage in daughter cells. In response to cellular stress, p53 induces the expression of downstream genes such as cyclin-dependent kinase (CDK) inhibitors, which regulate cell cycle checkpoint signals, causing the cell to undergo G_1 arrest and allowing DNA repair or apoptosis. Regulation of p53 can occur through MDM2, which reduces p53 levels through ubiquitination degradation. MDM2 in turn can be induced by p53 in a negative feedback loop or inhibited by the tumor suppressor $p14^{ARF}$ (encoded by *CDKN2A*). As such, *MDM2* and *CDKN2A* are commonly altered in lung cancer through amplification and loss of expression, respectively. p53 mutations are the most common alterations in lung cancer, where 17p13 frequently demonstrates hemizygous deletion and mutational loss of function of the remaining allele. Unlike most TSGs, which are predominantly inactivated by deletion or truncation, the majority of mutations in *TP53* are missense mutations. Most common are mutations in the DNA binding domain, which generally confer a loss-of-function phenotype by preventing p53 from binding to DNA and acting as a transcription factor. However, mutations in the homo-oligomerization domain can have a dominant negative effect, where mutant p53 exerts a dominant-negative effect on the remaining wild-type protein, abrogating the ability of wild-type p53 to inhibit cellular transformation. Because of the prevalence of p53-inactivating mutations in human cancers, large-scale efforts have been focused on therapeutic strategies to restore normal p53 function. These include re-introduction of wild-type p53 using gene therapy, pharmacological rescue of mutant p53 with small-molecule agents and peptides, blocking of MDM2 expression, inhibiting MDM2 ubiquitin ligase activity, and targeting the p53-MDM2 interaction with small-molecule inhibitors.

The $p16^{INK4a}$-RB Pathway

The $p16^{INK4a}$-RB1 pathway controls G_1-to-S-phase cell cycle progression. Hypophosphorylated retinoblastoma (RB) protein, encoded by *RB1*, was the first tumor suppresser gene identified in lung cancer and halts G_1/S phase transition by binding to the transcription factor E2F1 and repressing the transcription of necessary genes. RB is inhibited by hyperphosphorylation by CDK-CCND1 complexes (complexes between CDK4 or CDK6 and CCND1), and in turn, formation of CDK-CCND1 complexes can be inhibited by p16 (encoded by *CDNK2A*). Absent or mutant RB protein is found in approximately 90% of SCLCs compared to only 10% to 15% of NSCLCs, in which abnormalities in p16 are more common. Other components of the CDKN2A/RB pathway are also commonly altered in lung cancer through mutations (*CDK4* and *CDKN2A*), deletions (*RB1* and *CDKN2A*), amplifications (*CDK4* and *CCDN1*), methylation silencing (*CDKN2A* and *RB1*), and phosphorylation (RB).

Hallmark: Enabling Replicative Immortality

The enzyme telomerase prevents loss of telomere ends beyond critical points and is essential for cell immortality. Although silenced in normal cells (except stem cells), telomerase is activated in more than 80% of NSCLCs and almost uniformly in SCLCs, making it an attractive therapeutic target. Approaches to telomerase inhibition include using antisense oligonucleotides that bind to human telomerase RNA (such as imetelstat, which has started Phase II trials) and immunotherapy, in which a patient's immune system is stimulated with a vaccine to recognize tumor cells containing a major histocompatibility complex–presenting hTERT peptide on the cell surface.

Hallmark: Inducing Angiogenesis

The tumor microenvironment describes the complex and dynamic milieu of stromal cells that surround tumor cells. Cells that make up the tumor microenvironment interact both with each other and with tumor cells. As a consequence, they can affect tumor growth, invasion, and metastasis. Modulation of critical tumor microenvironment biomarkers could improve the current treatment of lung cancers.

Angiogenesis is one of the hallmarks of cancer, being essential for a microscopic tumor to expand into a macroscopic, clinically relevant tumor. A number of angiogenic proteins have been characterized, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin-8, and angiopoietins 1 and 2; most have been found to be dysregulated in some lung cancers. VEGF signaling is stimulated by tumor hypoxia, growth factors and cytokines, and oncogenic activation. VEGF is an important inducer of angiogenesis and is known to stimulate proliferation and migration, inhibit apoptosis, promote survival, and regulate endothelial cell permeability. It is highly expressed in both NSCLC and SCLC and is associated with poor prognosis in NSCLC. Two main approaches to anti-VEGF therapy are blocking VEGF from binding to its extracellular receptors using VEGF-specific antibodies and recombinant fusion proteins, or using small-molecule TKIs that bind to the intracellular region of VEGFR. The humanized monoclonal antibody bevacizumab blocks the binding of VEGF-A to its receptors VEGFR1 and VEGFR2 and is now approved for use in lung cancer. Interestingly, VEGF expression does not always correlate with response to bevacizumab, possibly because of SNPs in *VEGF*.

Hallmark: Activation Invasion and Metastasis

Epithelial-to-mesenchymal transition (EMT), involved in embryogenesis and normal development of multiple tissues and organs, has been implicated in tumor progression and metastasis.[15] EMT describes the loss of cell polarity into a motile, mesenchymal phenotype typically characterized by loss of E-cadherin expression. Conversion of epithelial tumor cells to a mesenchymal state promotes motility and invasiveness, allowing the tumor cells to detach from the primary tumor and relocate to a secondary site. Tumor cells then undergo a mesenchymal-to-epithelial transition (MET) to revert to an epithelial state to enable proliferative growth. Although EMT is involved in invasion and metastasis, it is also associated with early events in carcinogenesis, the acquisition of stem cell–like properties, and resistance to cell death, senescence, and conventional chemotherapies. In lung cancer, tumors expressing mesenchymal markers and EMT inducers (e.g., Vimentin, Twist, and Snail) have poor prognosis.[16] EMT has also been linked to lung cancer resistance to EGFR TKIs whereas COX-2 expression and loss of LKB1 have been shown to promote EMT in lung cancer. The miR-200 family of miRNAs is an important negative regulator of EMT and, as discussed previously, miR-200 expression is frequently lost in lung cancer, resulting in EMT.

Lung Cancer Stem Cells

The cancer stem cell (CSC) model hypothesizes there is a population of rare, stem-like tumor cells capable of self-renewing and undergoing asymmetric division, thereby giving rise to differentiated progeny that form the bulk of the tumor. The first evidence for CSCs (also termed *tumor-initiating cells*) was reported in acute myeloid leukemia, but support for their existence in solid tumors, including lung cancer, is becoming increasingly common.[17] Although identification of lung CSCs is technically challenging, several cell surface biomarkers have been reported for the detection and isolation of putative lung CSCs. It is also likely that markers of lung CSCs will differ between lung cancers, potentially related to lung cancer oncogenotype. Regulation of lung CSCs is likely by the Hedgehog (Hh), Wnt, and Notch stem cell signaling pathways. Normally tightly regulated processes important in normal lung development, genes whose products make up these pathways are often dysregulated or mutated in human cancers, including SCLC and NSCLC. The Wnt pathway has critical roles in organogenesis, cancer initiation and progression, and maintenance of stem cell pluripotency. It is suggested to be one of the most important signaling pathways in lung cancer as evidenced by dysregulation of many pathway members. Canonical Wnt signaling results in nuclear accumulation of β-catenin, causing transcriptional activation of many target genes. During embryogenesis, the Hh pathway is involved in organ development and body patterning, whereas in adults it is primarily activated during tissue repair. Activation of the Hh pathway has been reported in both NSCLC and SCLC. Notch signaling is important in cell fate determination and can promote and maintain survival in many human cancers; dysregulated Notch pathway components are therapeutic targets in lung CSCs.

CSCs are thought to have higher resistance to cytotoxic therapies and radiotherapy than the bulk tumor cells and contribute to tumor recurrence, leading to approaches to specifically treat the CSC population through inhibition of important signaling pathways. Specific inhibitors of Hh and Notch signaling have shown efficacy in lung cancer preclinical models and are now in clinical trials (see Table 32-4).

Lineage-Dependent Oncogenes: *SOX2* and *NKX2-1* (*TITF1*)

Genome-wide screens for DNA copy number changes in primary NSCLCs has led to the identification of recurrent, histologic subtype-specific focal amplification at 14q13.3 (*NKX2-1 (TITF1)*) (adenocarcinoma) and 3q26.33 (*SOX2*) (SCC). Functional analysis identified *NKX2-1* (also termed *TITF1*) and *SOX2* as the respective targets of these

amplifications. Amplification of tissue-specific transcription factors in cancer has been previously observed in other cancers, leading to the development of a "lineage-dependency" concept in tumors[18] where the survival and progression of a tumor is dependent on continued signaling through specific lineage pathways (i.e., abnormal expression of pathways involved in normal cell development) rather than continued signaling through the pathway of oncogenic transformation, as seen with oncogene addiction.

NKX2-1 encodes a lineage-specific transcription factor essential for branching morphogenesis in lung development and the formation of type II pneumocytes—the cells lining lung alveoli. Initial studies reported on the oncogenic role of *NKX2-1* in lung adenocarcinoma; however, recent in vivo data suggest that it also has a tumor-suppressive role by promoting differentiation and suppressing metastasis. In patients with advanced lung adenocarcinoma, patients with NKX2-1–negative tumors had poorer survival. ROR1 has been shown to be a direct transcriptional target of NKX2-1 and is crucially involved in sustaining a favorable balance between pro-survival PI3K-AKT signaling and the pro-apoptotic p38 pathway.

Sex determining Y-box 2 (SOX2) amplification was identified specifically in SCCs and is required for normal esophageal squamous development. Together with Oct4, Klf4, and c-Myc, Sox2 comprises one of the four "Yamanaka" transcription factors that are able to reprogram differentiated cells into induced pluripotent stem cells (iPSCs). SOX2 has been shown to have a tumor- and metastasis-promoting role in lung cancer and is implicated in the early pathogenesis of lung SCC. In addition, genome-wide analyses have identified SOX2 as frequently overexpressed by several genetic mechanisms in SCLC. Knockdown studies show it has a direct functional role in the growth of SCLC.[25]

Preclinical Model Systems for Lung Cancer

Although genome-wide approaches have the capacity of identifying novel genes or interactions in relation to lung cancer, the functional relevance of these findings needs to be characterized in preclinical model systems of lung carcinogenesis. Lung cancer cell lines, cell-line xenografts (implantation of cell lines into immunocompromised mice), and patient-derived xenografts (direct implantation of small tumor fragments into immunocompromised mice) are important models of spontaneously occurring lung cancer and enable analysis of response to therapeutic agents. Furthermore, patient-derived xenografts also provide a realistic representation of tumor cell subpopulation heterogeneity and tumor microenvironment (at least in early passages). However, lung cancers and their derived cell lines and xenografts usually have hundreds to thousands of genetic and epigenetic changes. By contrast, two much simpler and more valuable models to study the progression of lung carcinogenesis are immortalized human bronchial epithelial cells (HBECs) and genetically engineered mouse models (GEMMs). These systems provide methods to reduce the inherent complexity and heterogeneity of the lung cancer genome and allow characterization of single or sequential genetic alterations in relation to the development, maintenance, and progression of lung cancer. HBECs are derived from primary human airway epithelial cells and immortalized with either viral oncoproteins (such as SV40 early region) or *Cdk4* with hTERT. These systems can model the stepwise oncogenic transformation of lung epithelial cells following the introduction of defined genetic manipulations commonly found in lung cancer. GEMMs allow the study of lung cancer pathogenesis with defined changes in the setting of the whole organism, and as with patient-derived xenografts, they provide a realistic representation of the tumor microenvironment. GEMMs were critical in developing our understanding of oncogene dependence, as observed in conditional $Kras^{D12}$-induced lung adenocarcinomas, where switching off the driving oncogene was sufficient to induce tumor regression even in the presence of other nondriving oncogenic alterations. Ensuing research has characterized several conditional lung tumor–inducing combinations of oncogenic activations in mice,[20] which have been used to test new targeted therapies, improve the effectiveness of conventional chemotherapies, identify biomarkers and imaging strategies for early detection, and study disease relapse and metastasis. Recently, GEMMs targeting oncogenic alterations to specific lung epithelial cell subpopulations has provided a clearer understanding of the specific cells giving rise to lung cancer.

Translation of Molecular Data to the Clinic: Rationale-Based Targeted Therapy

Characterization of the molecular changes in lung cancer and associated preneoplastic cells is becoming increasingly well defined, aided immeasurably by the continued advancement of both clinical and genomic tools. These advances promote our understanding of the development and progression of lung cancer, which is of fundamental importance for improving the prevention, early detection, and treatment of this disease. Ultimately these findings need to be translated to the clinic by using these molecular alterations as biomarkers for early detection and risk assessment; as targets for prevention; as signatures for personalizing

prognosis and therapy selection for each patient; and as therapeutic targets to allow selective killing or growth inhibition of lung cancer.

Improved detection and sampling of clinical samples using fluorescent bronchoscopy, endobronchial ultrasounds, and laser capture microdissection techniques, for instance, enables precise analysis of abnormal epithelial cells. Although some significant advancements have been targeted therapy (in EGFR mutant and EML4-ALK-positive lung tumors), we have yet to move any biomarkers for risk or early detection of lung cancer into clinical use. This chapter has outlined some of the significant molecular alterations known to be involved in the initiation and/or progression of lung cancer, but continued development of biomarkers and targeted therapies is dependent on increased understanding of involved molecules and pathways.

The recent rapid pace of progress in the field of genomics and bioinformatics now gives researchers the tools to correlate patient subsets with augmented sensitivity to conventional or targeted therapeutics, distinguish driver versus passenger mutations, and better focus the design on novel therapeutic targets. To achieve these goals, we will continue to need high-quality samples from patients with a wide variety of lung cancer types collected at initial diagnosis and at various points during disease progression; incorporation of comprehensive genomic studies in clinical trials with molecularly targeted agents; timely mutation testing of clinically available materials (such as FFPE specimens) using clinical laboratory practices (CLIA-certified laboratory methods); and a new cadre of clinical investigators conversant with cancer genomics trained to effectively translate these findings in the clinic. Finally, identifying and unraveling the intricate and interlinked pathways will require integrating laboratory and clinical investigations. The strong interplay among cancer genomics, bench research, and clinical trials will advance our understanding of lung cancer biology and lead to improved detection, diagnosis, treatment, and prognosis of lung cancer by achieving "personalized medicine," the selection of the best treatment for each patient based on tumor-associated biomarkers.

Acknowledgments

We thank the many current and past members of the Minna lab for their contributions to lung cancer translational research and our especially our long-term collaborator, Dr. Adi Gazdar. Also we apologize to other investigators for the omission of any references.

Key References

4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
6. Pao W, Hutchinson KE. Chipping away at the lung cancer genome. *Nat Med*. 2012;18:349-351.
7. Sriram KB, Larsen JE, Yang IA, et al. Genomic medicine in non-small cell lung cancer: paving the path to personalized care. *Respirology*. 2011;16:257-263.
8. Shedden K, Taylor JM, Enkemann SA, et al. Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nat Med*. 2008;14:822-827.
9. Hammerman PS, Lawrence MS, Voet D, et al. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012.
12. Larsen JE, Minna JD. Molecular biology of lung cancer: clinical implications. *Clin Chest Med*. 2011;32:703-740.
17. Alamgeer M, Peacock CD, Matsui W, et al. Cancer stem cells in lung cancer: Evidence and controversies. *Respirology*. 2013;18:757-764.
21. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers–a different disease. *Nat Rev Cancer*. 2007;7:778-790.
24. Imielinski M, Berger AH, Hammerman PS, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell*. 2012;150:1107-1120.
25. Rudin CM, Durinck S, Stawiski EW, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet*. 2012;44:1111-1116.
29. Larsen JE, Cascone T, Gerber DE, et al. Targeted therapies for lung cancer: clinical experience and novel agents. *Cancer J*. 2011;17:512-527.

This page intentionally left blank

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2012;2012(62):10-29.
2. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194:23-28.
3. Dakubo GD, Jakupciak JP, Birch-Machin MA, et al. Clinical implications and utility of field cancerization. *Cancer Cell Int*. 2007;7:2.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
5. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics. *CA Cancer J Clin*. 2002;2005(55):74-108.
6. Pao W, Hutchinson KE. Chipping away at the lung cancer genome. *Nat Med*. 2012;18:349-351.
7. Sriram KB, Larsen JE, Yang IA, et al. Genomic medicine in non-small cell lung cancer: paving the path to personalized care. *Respirology*. 2011;16:257-263.
8. Shedden K, Taylor JM, Enkemann SA, et al. Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nat Med*. 2008;14:822-827.
9. Hammerman PS, Lawrence MS, Voet D, et al. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012.
10. Kris MG, Johnson BE, Kwiatkowski DJ, et al. Identification of driver mutations in tumor specimens from 1000 patients with lung adenocarcinoma: The NCI's lung cancer mutation consortium (LCMC). *J Clin Oncol*. 2011:29.
11. Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. *Nature reviews. Cancer*. 2005;5:689-698.
12. Larsen JE, Minna JD. Molecular biology of lung cancer: clinical implications. *Clin Chest Med*. 2011;32:703-740.
13. Weinstein IB. Cancer. Addiction to oncogenes–the Achilles heel of cancer. *Science*. 2002;297:63-64.
14. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol*. 2005;6:635-645.
15. Thiery JP, Acloque H, Huang RY, et al. Epithelial-mesenchymal transitions in development and disease. *Cell*. 2009;139:871-890.
16. Miura N, Yano T, Shoji F, et al. Clinicopathological significance of Sip1-associated epithelial mesenchymal transition in non-small cell lung cancer progression. *Anticancer Res*. 2009;29:4099-4106.
17. Alamgeer M, Peacock CD, Matsui W, et al. Cancer stem cells in lung cancer: Evidence and controversies. *Respirology*. 2013;18:757-764.
18. Garraway LA, Sellers WR. Lineage dependency and lineage-survival oncogenes in human cancer. *Nat Rev Cancer*. 2006;6:593-602.
19. REFERENCE DELETED IN PROOFS
20. Farago AF, Snyder EL, Jacks T. SnapShot: Lung cancer models. *Cell*. 2012;149 246-246 e1.
21. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers–a different disease. *Nat Rev Cancer*. 2007;7:778-790.
22. Rudin CM, Avila-Tang E, Harris CC, et al. Lung cancer in never smokers: molecular profiles and therapeutic implications. *Clin Cancer Res*. 2009;15:5646-5661.
23. Subramanian J, Govindan R. Lung cancer in never smokers: a review. *J Clin Oncol*. 2007;25:561-570.
24. Imielinski M, Berger AH, Hammerman PS, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell*. 2012;150:1107-1120.
25. Rudin CM, Durinck S, Stawiski EW, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet*. 2012;44:1111-1116.
26. Suzuki M, Yoshino I. Aberrant methylation in non-small cell lung cancer. *Surg Today*. 2010;40:602-607.
27. Heller G, Zielinski CC, Zochbauer-Muller S. Lung cancer: from single-gene methylation to methylome profiling. *Cancer Metastasis Rev*. 2010;29:95-107.
28. Wen J, Fu J, Zhang W, et al. Genetic and epigenetic changes in lung carcinoma and their clinical implications. *Mod Pathol*. 2011;24:932-943.
29. Larsen JE, Cascone T, Gerber DE, et al. Targeted therapies for lung cancer: clinical experience and novel agents. *Cancer J*. 2011;17:512-527.
30. Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer*. 2011;11:849-864.
31. Enfield KS, Pikor LA, Martinez VD, et al. Mechanistic Roles of Noncoding RNAs in Lung Cancer Biology and Their Clinical Implications. *Genet Res Int*. 2012;2012:737416.
32. Du L, Pertsemlidis A. microRNAs and lung cancer: tumors and 22-mers. *Cancer Metastasis Rev*. 2010;29:109-122.
33. Fanini F, Vannini I, Amadori D, et al. Clinical implications of microRNAs in lung cancer. *Semin Oncol*. 2011;38:776-780.
34. Liu X, Sempere LF, Guo Y, et al. Involvement of microRNAs in lung cancer biology and therapy. *Transl Res*. 2011;157:20020-20028.

Matthew L. Hedberg and Jennifer R. Grandis

33

The Molecular Pathogenesis of Head and Neck Cancer

Epidemiology and Clinical Considerations

Head and neck squamous cell carcinoma (HNSCC) arises in the oral cavity, oropharynx, hypopharynx, and larynx. It accounts for more than 90% of the cancers of the head and neck and is the sixth most common cancer by incidence worldwide. In the United States, approximately 40,250 new cases of HNSCC were expected in 2012, with the incidence in men being more than twice the incidence in women. Rates of death due to HNSCC have declined only slightly in the United States over the past 3 decades, and the all-stage survival rates of 61% and 50%, at 5 and 10 years, respectively, illustrate the need for improved therapy.[1-3]

Tobacco use and alcohol consumption are the two most important risk factors for the development of HNSCC, and their contributions to risk are synergistic. In addition, the human papillomavirus (HPV), especially HPV-16, is now a well-established independent risk factor. Present in approximately 20% of all HNSCC, HPV is believed to be responsible for more than 60% of oropharyngeal SCC, specifically, from 2004 to 2008.[4,5] HPV(+) HNSCC is currently considered, based on clinicopathological and molecular characterizations, to be a unique subtype of HNSCC and has been shown to generally be associated with a favorable prognosis, compared to HPV(−) subtypes.[6,7] Greatly increased susceptibility to HNSCC is seen in some heritable conditions of impaired genome maintenance, such as Fanconi anemia.[8] The Epstein-Barr virus is a known risk factor for nasopharyngeal carcinoma.[9]

Stage at presentation correlates strongly with prognosis; HNSCC is staged via the tumor, node, metastasis (TNM) staging system.[10] It has also been demonstrated, however, that HPV status and tobacco use are of important prognostic value as well, offering potentially greater predictive value than the traditional TNM staging in the case of oropharyngeal cancers.[6] The field cancerization theory, proposed in 1953 by Slaughter and colleagues to describe the developmental pattern of invasive HNSCC from a precancerous field of atypical mucosal epithelium as depicted in Figure 33-1, has been implicated in the high incidence of recurrence and second primary tumor formation. Early-stage tumors generally have a favorable prognosis and are treated with surgery or radiotherapy. Advanced-stage tumors are treated with a combination of surgery, radiation, and chemotherapy, with primary surgery for oral cavity tumors followed by adjuvant radiation or chemoradiation, and organ-preservation protocols with combined chemoradiation for pharyngeal and laryngeal cancers. Recently, the use of targeted therapeutics has emerged in HNSCC treatment. The most mature target to date is the epidermal growth factor receptor (EGFR). Anti-EGFR antibodies, in combination with traditional radiotherapy, have demonstrated increased overall survival and progression-free survival in the newly diagnosed setting and improved survival with chemotherapy in the recurrent/metastatic setting.[11,12]

Oncogenic Progression of HNSCC

The well-documented histological progression of HNSCC from oral leukoplakia through progressive phases of hyperplasia, dysplasia, carcinoma in situ, and ultimately invasive carcinoma is believed to correspond with the accumulation of genetic alterations.[13] In HNSCC, one of the earliest initiating events is likely the clonal proliferation of precancerous cells with *TP53* mutations.[14] This is followed by the accumulation of additional genetic alterations within clonal subpopulations, in an order that is not well defined and very likely varies among patients. A hypothetical model of development and genetic progression of the primary subtypes of HNSCC is presented in Figure 33-2. Recently, whole-exome sequencing studies of HNSCC have begun to reveal the genetic underpinnings of this disease. These data, in combination with previous genomic analyses, have identified the most commonly mutated genes in HNSCC as outlined in Table 33-1.[15,16]

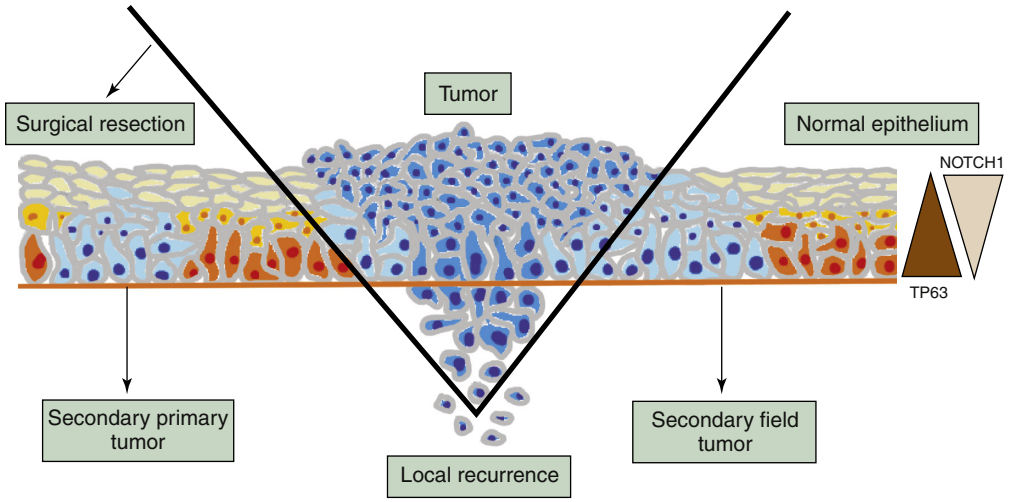


Figure 33-1 The Field Cancerization Theory is defined as the presence of one or more mucosal areas consisting of epithelial cells with cancer-associated genetic or epigenetic alterations. A precursor field *(light blue)* is monoclonal but does not show invasive growth or metastatic behavior, which are the hallmarks of an invasive carcinoma *(dark blue)*. A field is preneoplastic by definition; it may or may not have histological alterations characteristic of dysplasia.[4] A leukoplakia is the clinical manifestation of a field, though most fields are clinically invisible. Additional genetic changes are needed to transform a field into a carcinoma. The field and primary tumor share genetic alterations and have a common clonal origin. Clinically, a field may be the source of local recurrences, second field tumors, and second primary tumors after surgical resection of the initial carcinoma. These legions are distinguished on the basis of their distance from the index tumor, or the time interval after which they develop. A local recurrence *(lower center)* arises from residual tumor cells and is less than 2 cm away from, and/or occurs within 3 years of, the primary tumor. A second primary tumor *(lower left)* is more than 2 cm away from, and/or occurs more than 3 years after, the primary tumor. Tumors that arise from a contiguous portion of the same field that gave rise to the original primary tumor have been described as second field tumors *(lower right)*.[4] Studies attempting to identify specific genetic characteristics that determine the risk of a field developing into cancer have shown that genetic changes at chromosome 9p, decreased cytokeratin 4 expression, and decreased cornulin expression are potential biomarkers.[69,70] Leukoplakia studies have demonstrated that the presence and number of genetic changes, typically chromosome 9p loss, chromosome 3p loss, and chromosome 17p loss, are associated with the risk of progression.[4] At *far right*, the *TP63/NOTCH1* expression gradient of normal epithelium is illustrated. Perturbation of this gradient is believed to be a component of precancerous fields and invasive HNSCC legions. The normal process of squamous differentiation in mucosa is controlled in part by *TP63* and *NOTCH1*. *TP63* is expressed in keratinocytes of the basal layer, where it maintains their proliferative potential and regulates expression of basal keratins. Expression of *NOTCH1* results in terminal differentiation of cells in the spinous and granular layers expressing alternative keratins.[44,46-48,52,53] *(Adapted from C. René Leemans, Boudewijn J. M. Braakhuis, and Ruud H. Brakenhoff. (2010). The molecular biology of head and neck cancer. Nature Reviews Cancer. doi:10.1038/nrc2982.)*

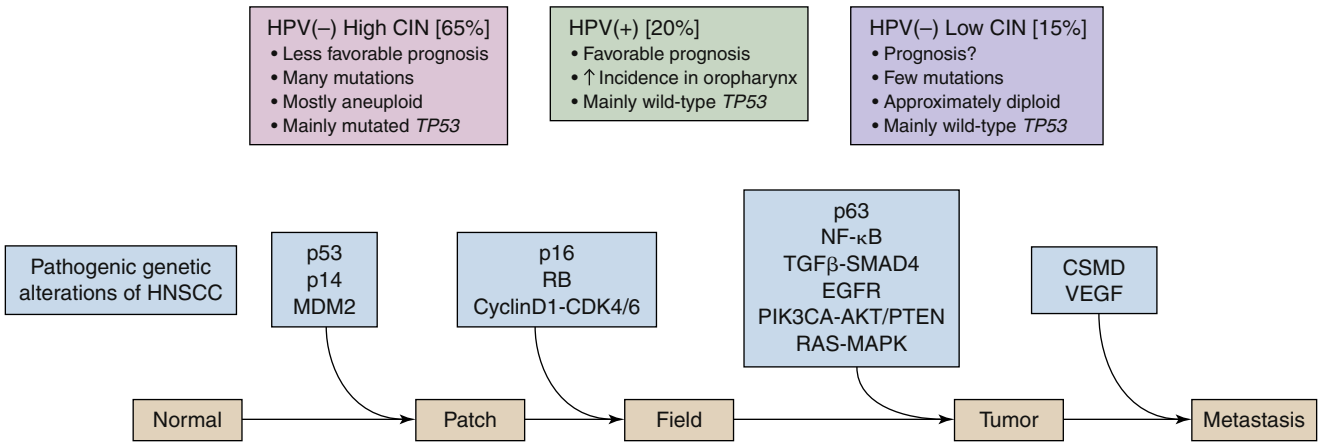


Figure 33-2 A hypothetical model of HNSCC development depicting the genetic alterations implicated in the process by current data. The model is a generalization and thus is varyingly accurate among subtypes of HNSCC. Three steps are critical in this model: A progenitor or adult stem cell acquires one (or more) genetic alterations, usually including an alteration of p53, and forms a patch containing clonal, genetically altered daughter cells. Then, by escaping normal growth control and/or gaining growth advantage, this clonal patch develops into an expanding field. Eventually, through a further accumulation of genetic alterations, a subclone in the field evolves into an invasive cancer and progresses to metastasis. Both aneuploidy and the accumulation of cancer-associated genetic changes in fields are linked to the risk of malignant progression. In addition, the three main clinicopathologic subtypes of HNSCC are depicted: HPV(+) HNSCC, HPV(–) HNSCC with many numerical genetic changes (high CIN), and HPV(–) HNSCC with few genetic changes (low CIN).[4] Although drawn as distinct steps for the purpose of illustration, the actual order of acquisition of distinct alterations is not known at this time. *CDK,* Cyclin-dependent kinase; *CSMD*, CUB and SUSHI multiple domain protein; *NF-κB,* nuclear factor-κB; *PIK3CA,* phosphoinositide-3 kinase subunit-α; TGFβ, transforming growth factor-β; *VEGF*, vascular endothelial growth factor. *(Adapted from C. René Leemans, Boudewijn J. M. Braakhuis, and Ruud H. Brakenhoff. (2010). The molecular biology of head and neck cancer. Nature Reviews Cancer. doi:10.1038/nrc2982.)*

Table 33-1 Common Genetic Alterations in HNSCC

Gene*	Description	Mutated (Activating/Missense/Inactivating)
TP53[a,b]	Transcription factor	53% (NA/28%/25%)
CCND1[b]	Cell cycle activator (Cyclin D1)	25% (25%/NA/NA)
CDKN2A[a,b]	Cell cycle inhibitor/p53 activator	21% (NA/3%/18%)
NOTCH1[a,b,*]	Receptor/transcription factor	15% (NA/8%/8%)
CSMD3[a,b]	Putative adhesion factor	13% (NA/12%/1%)
USH2A[a,b]	Basement membrane protein	12% (NA/11%/1%)
PIK3CA[a,b,*]	PI3 kinase catalytic subunit	10% (7%/7%/NA)
PRDM9[b,*]	Histone methyltransferase	8% (NA/8%/1%)
COL22A1[b,*]	Pro-apoptotic effector	8% (NA/7%/2%)
RIMS2[b,*]	Putative synaptic vesicle regulator	8% (NA/8%/NA)
ZFHX4[b]	Zinc finger homeodomain	8% (NA/8%/NA)
MLL2[b,*]	Histone methyltransferase	8% (NA/4%/4%)
NAV3[b]	Axonal/cytoskeleton guide	8% (NA/7%/1%)
CASP8[a.b]	Pro-apoptotic proteolyase	7% (NA/2%/5%)
TP63[a,b]	Transcription factor	7% (NA/5%/1%)
NSD1[b]	Histone methyltransferase	7% (NA/3%/4%)
EGFR[b]	Growth factor Rtk	7% (7%/NA/NA)
PTEN[b]	Lipid phosphatase—PI3K inhibitor	6% (NA/3%/3%)
FBXW7[a,*]	Ubiquitin ligase	5% (NA/3%/2%)
HRAS[a,b,*]	RTK signaling protein	4% (4%/4%/NA)
IRF6[b]	Interferon regulatory factor	4% (NA/3%/1%)
NOTCH2[b]	Receptor/transcription factor	4% (NA/3%/1%)
NOTCH3[b,*]	Receptor/transcription factor	4% (NA/2%/2%)

Displays the percentage of samples in the [a]Agrawal et al.[15] and [b]Stranksy et al.[16] exome sequencing studies with at least one mutant allele or copy number loss/amplification of a given gene. It also identifies the percentage of each respective mutation that is activating (known activating mutation or copy number amplification), missense, and inactivating (nonsense, splice site, frame shift, insertion/deletion, or copy number loss).
*Mutations present in at least one HPV(+) tumor.

Molecular Pathogenesis of HNSCC: Interfacing Genomic Pathways

Conceptually, there are six major hallmarks that define the current understanding of a cancerous cellular phenotype: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.[17] Research to date indicates that the altered oncogenes and tumor suppressors of HNSCC act primarily in functional pathways known to largely determine cellular proliferation, cell survival, squamous epithelial differentiation, and invasion and metastasis. Individual genes may function in more than one pathway, and the pathways themselves interface with, and influence, each other, as depicted in Figure 33-3. The following sections discuss some of the most common gene alterations believed to contribute to HNSCC oncogenesis in the context of these pathways.

Cell Cycle and Proliferation: TP53/CDKN2A/RB/CCND1/TERT

Though oncogenes have been identified, the high mutation rates seen in most cases of HNSCC, along with the recent exome sequencing data, suggest that loss-of-function mutations in tumor suppressor genes represent the predominant genetic pathology observed in HNSCC.[4,15,16] Foremost among these tumor suppressors is the *TP53* gene product: p53. A nuclear phosphoprotein that signals through its key

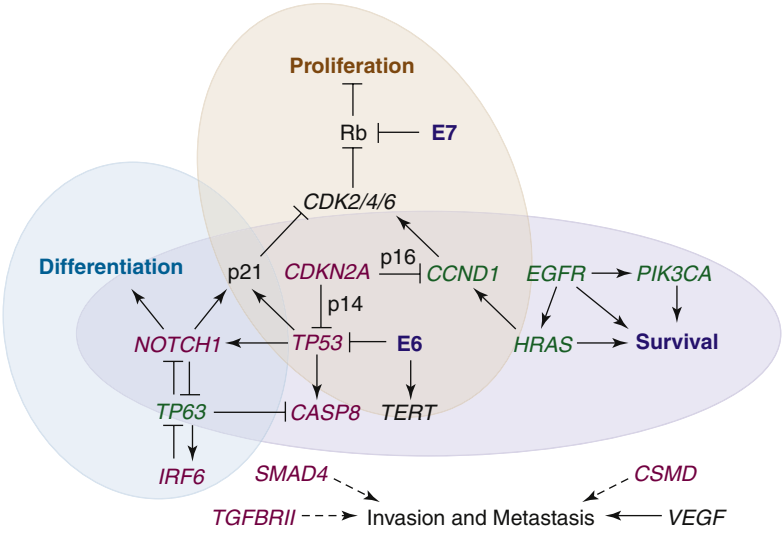


Figure 33-3 The interfacing genetic alterations of putative oncogenes *(green)*, tumor suppressors *(red)*, and signaling pathways that mediate the hallmarks of HNSCC. Loss of *TP53* and *CDKN2A*, either through mutation or expression of the HPV E6 and E7 proteins *(blue)*, along with amplification of *CCND1* favors survival and permits proliferation through the increased activity of cyclin-dependent kinases (CDKs) and loss of p53-dependent apoptosis. Although intact differentiation programs and alternative apoptotic programs may restrict abnormal cell cycling for a time, loss of *NOTCH1* and/or abnormal expression of *TP63*, along with the acquisition of alterations in other survival genes, such as *CASP8*, *PIK3CA*, and *EGFR*, remove additional barriers to tumor cell proliferation and survival. Upregulation of pro-angiogenic genes permits the growth of tumors, and the loss of cell adhesion genes allows for the release of cells from the mucosal lining. Invasion through the basement membrane is promoted by *TGFβ-SMAD* signaling, the loss of which initially contributes to tumorigenesis, and whose later reactivation drives metastasis. Several genes and signaling pathways, including *TP53*, *TP63*, and *NOTCH1*, contribute to more than one hallmark by influencing each other's expression and/or activity.

downstream partner, cyclin-dependent kinase inhibitor 1 (p21), p53 can influence both the G_1 and G_2 checkpoints of the cell cycle, although it is traditionally thought of as being the primary G_2 checkpoint regulator. Canonically, in response to DNA damage, p53 activation inhibits cell cycle progression and prevents apoptosis, allowing the cell time to repair the damaged DNA. If the DNA damage cannot be repaired, apoptosis ensues. Loss of p53 function allows cells with damaged DNA to proliferate freely, resulting in the accumulation of potentially oncogenic mutations in the genome of affected cells.

Mutation of *TP53* is the earliest and most frequent mutation event observed in HPV-negative HNSCC. Occurring in more than 50% of cases, mutation is significantly associated with decreased survival.[18] The majority of *TP53* mutations are found in exons 5 through 9, the DNA binding region, with mutations at several specific codons known to be associated with tobacco exposure.[7] In a large portion of HNSCC without somatic *TP53* mutations, the activity of p53 is compromised by other mechanisms, including E6 expression in HPV(+) cancers, which inactivates p53, and overexpression and/or amplification of *MDM2*, which promotes the degradation of p53.

Chromosomal loss of 9p21 has been reported in 70% to 80% of dysplastic oral mucosa legions progressing to HNSCC.[19] This illustrates an interface between pathways, as the *CDKN2A* locus is found within 9p21. Two *CDKN2A* protein products, $p16^{INK4A}$ and $p14^{ARF/INK4B}$, are involved in cell cycle regulation. Specifically, $p14^{ARF/INK4B}$ is known to downregulate *MDM2*, thereby regulating p53 levels.[20] In all, p53 function is believed to be downregulated through one or more mechanisms in at least 80% of HNSCC.[4]

A second *CDKN2A* transcript, $p16^{INK4A}$, implicates the Retinoblastoma (*RB*) pathway, which is the primary G_1 checkpoint regulator, in HNSCC. $p16^{INK4A}$ inhibits the Cyclin D1/Cyclin-Dependent Kinase (CDK) complex, which normally functions to inactivate *RB*-encoded pocket proteins via phosphorylation, allowing for the dissociation and activation of Elongation Factor-2 and subsequent entry into S phase. Inactive, phosphorylated *RB* pocket proteins are unable to block the G_1-to-S phase transition in the setting of $p16^{INK4A}$ loss.[20] In addition to chromosomal loss of 9p21, recent studies have demonstrated *CDKN2A* mutations in approximately 7% of tumors and copy number losses in another 20% to 30%.[15,16] The mechanism of $p16^{INK4A}$ loss has been shown to be of prognostic value in oral SCC: epigenetic silencing was found to be associated with higher recurrence rates, and deletion with increased rates of nodal metastases.[21] Analogous to the inhibition of p53 by HPV E6 expression, E7 expression in HPV(+) HNSCC inactivates the *RB* pathway by binding RB1. Because E7 expression can inhibit the *RB* pathway, there is less selective pressure for $p16^{INK4A}$ loss in HPV(+) HNSCC. As a result, immunohistochemical staining for $p16^{INK4A}$ is used clinically in establishing the HPV status of HNSCC, along with polymerase chain reaction (PCR)-based methods.[22]

Further evidence of the important role played by the *RB* pathway in HNSCC is that the commonly found amplification of 11q13, which contains *CCND1*, in combination with other potential mechanisms, results in the overexpression of Cyclin D1 in up to 80% of HPV(−) tumors.[4]

Intriguingly, *CDKN2A* loss and *CCND1* gain, though seemingly redundant mechanisms to evade the G_1 checkpoint, are not mutually exclusive events in HNSCC. Both occur frequently and remain under investigation as independent and synergistic markers of poor prognosis. [23] Cyclin D1 has been found to sequester certain *CDK* inhibitors and to bind transcription factors such as PPARγ, and various DNA repair proteins such as Rad51.[24] It remains to be established whether any of these interactions contributes to a noncanonical consequence of *CCND1* functional loss in HNSCC.

Finally, the role of telomerase in promoting limitless replicative potential must be considered. The activity of telomerase (TERT) is detectable by immunostaining in approximately 80% of HNSCC cases analyzed. In most in vitro HNSCC models, TERT activity is necessary for immortalization of cell lines. However, keratinocytes may elongate their telomeres in a TERT-independent fashion, and *TERT* (5p15.33) is not known to be frequently gained or amplified in HNSCC. Although the exact role of *TERT* is still unclear, it remains a candidate cancer gene in HNSCC.[4]

Apoptosis and Survival: EGFR/RAS-MAPK/PIK3CA-AKT/CASP8

Cell cycle alterations, reduced immunogenicity, promotion of angiogenesis, and inhibition of apoptosis are just some of the many mechanisms underlying the enhanced survival of HNSCC. These cancerous traits are generated by genetic and epigenetic alterations in several pathways. Of particular importance in HNSCC are the receptor tyrosine kinase (RTK)-based signaling pathways. The class 1a phosphatidyl-inositol-3 kinases (PI3K) are heterodimers coupled to RTKs, such as the EGFR, or adaptor molecules. The PI3K-AKT kinase (AKT) signaling pathway mediates resistance to apoptosis and survival. Activated PI3K generates the lipid second-messenger phosphatidylinositol-3,4,5-P3 (PIP3), which serves to activate AKT. AKT is a serine/threonine kinase that, when activated, phosphorylates many downstream transcription factors, apoptosis inhibitors, cell cycle inhibitors, and other proteins, ultimately promoting cell survival and proliferation. This pathway is held in check by the action of the tumor suppressor phosphate and tensin homologue (PTEN), which dephosphorylates PIP3, thereby deactivating AKT. If PTEN activity is compromised, PI3K-AKT signaling can be irreversibly activated by RTK stimulation.[25]

Inactivating *PTEN* mutations have been reported in approximately 10% of HNSCC, PTEN expression is undetectable in nearly 30% of tongue cancers, and loss of heterozygosity of the *PTEN* locus has been observed in up to 40% of HNSCC.[26] Furthermore, recent evidence suggests that loss of even a single *PTEN* allele can contribute to tumorigenesis.[27] Three different "hotspot" activating mutations have been reported in *PI3KCA*, which codes for the catalytic subunit of PI3K.[28] Notably, the frequency of *PI3KCA* mutations is higher in HPV(+) HNSCC, suggesting a possible interaction between the PI3K pathway and the E6/E7 proteins of HPV. This has been suggested to be contributory to the development of invasive SCC in cervical cancer.[15,16,29] The PI3K-AKT axis is of consequence therapeutically in HNSCC as well, with numerous targeted inhibitors now in clinical trials.[29,30]

RAS family GTPases (HRAS, KRAS, and NRAS) are molecular switches that cycle between two conformational states: an active GTP-bound form, and an inactive GDP-bound form. The first RAS effector pathway to be identified was the RAS-RAF-MEK-MAPK pathway. The pathway is a common and essential element of mitogenic signaling driven by RTKs, resulting in a diverse array of cellular responses. RAF proteins are serine/threonine kinases that bind to the effector region of RAS-GTP. This interaction induces translocation of the protein to the plasma membrane. There, RAF proteins are activated and phosphorylated by different protein kinases. Active RAF phosphorylates MEK that, in turn, phosphorylates and activates MAPK. Activated MAPK serves as the terminal effector of the pathway, influencing cellular growth, differentiation, inflammation, apoptosis, and senescence. Mutant RAS, in which it assumes a permanently active conformation, is a well-established oncogene, found in approximately 25% of human tumors.[31]

HNSCC is unique in that *HRAS* mutations, being found in 3% to 5% of HNSCC, are more prevalent than *KRAS* or *NRAS* mutations.[15,16] These *HRAS* mutations are known to be associated with HNSCC in smokers, and in mouse models exposed to chemical carcinogens.[32] The exact contribution of the *HRAS* mutations to oncogenesis has yet to be elucidated in HNSCC. The RAS-MAPK and PI3K-ATK pathways interact directly and indirectly, through multiple intermediates.[31] In addition, *HRAS* mutations have been detected in HPV(+) tumors, allowing for the possibility of cooperation with oncogenic viral proteins.[15,16] Recent in vitro evidence suggests that even a single *HRAS* mutation, in the background of HPV and *MYC* alteration, can contribute to tumorigenesis.[33] Although the success of therapies targeting RAS proteins has been limited to date, several attempts to target their downstream effectors have shown promising results in preclinical models.[34]

RTKs lie upstream of both the RAS-MAPK and PI3K-ATK pathways. Most importantly for HNSCC, is *EGFR* (7p12), which codes for the prototypical ErbB family Type I RTK. Signaling through EGFR represents another interface between pathways, as it is involved in a variety of cellular processes, including survival and differentiation. EGFR has an extracellular ligand-binding domain, a transmembrane portion, and an intracellular kinase domain with five autophosphorylation sites. Ligand binding by EGFR monomers drives homodimerization or heterodimerization with another RTK, resulting in the initiation of downstream survival and proliferation signaling pathways. Two important and well-studied pathways activated by EGFR ligand binding are the RAS-MAPK and PI3K-ATK pathways. These independent cascades converge via the ultimate upregulation of Cyclin D1. Furthermore, when bound to EGF, EGFR itself can translocate to the nucleus, where it acts as a transcription factor for several genes including *CCND1*, and as a co-activator for other transcription factor proteins, such as the STAT proteins.[4]

EGFR is expressed in most epithelial tissues, and its dysregulation has been repeatedly shown to contribute to epithelial oncogenesis. In HNSCC, EGFR expression levels are nearly ubiquitously elevated in tumor and tumor-adjacent tissue compared to corresponding normal mucosa. Higher expression levels and copy number gain correlate with decreased survival but have not been highly indicative of improved response to EGFR-directed therapy. There are three FDA-approved EGFR targeting agents in clinical use: gefitinib and erlotinib, both TKIs, and cetuximab, a monoclonal antibody against EGFR, which is the only agent approved for use in HNSCC. All have shown modest efficacy as monotherapies to date, with EGFR-targeted therapies being effective in about 20% of patients in large multicenter trials, generally in combination with radiation and/or chemotherapy.[35] Expression of EGFRvIII, an *EGFR* allele harboring a large in-frame deletion of exons 2 through 7, can confer resistance to anti-EGFR therapy. The prevalence of the EGFRvIII variant remains controversial in HNSCC, with various studies reporting its expression to be present in anywhere from 0% to 42% of the tumors assayed.[36-39] Investigations into EGFRvIII mechanism(s) of oncogenesis continue, as therapies specifically directed against EGFRvIII have shown promise in glioblastoma and may be applicable in refractory HNSCC.[39] Another genetic alteration, reported in some cases of HNSCC, that is believed to contribute to anti-EGFR therapy resistance is mutation or amplification of the *MET* gene, which codes for another RTK.[40,41] *MET* has been implicated as a cancer gene in HNSCC that influences cell growth, motility, and angiogenesis.[4] This, too, may be of particular clinical consequence because there are both monoclonal antibodies and small-molecule inhibitors, FDA-approved in other cancers, with the ability to inhibit MET kinase activity.[42,43]

Finally, in addition to the growth factor signaling pathways that indirectly influence apoptosis, recent studies in HNSCC have found alterations directly within the apoptosis cascade itself. *CASP8*, a proteolyase responsible for initiating the caspase cascade that drives apoptosis, was found to be mutated in 8% of HNSCC by exome sequencing; *BCL2*, which prevents apoptosis, has been observed to be overexpressed in some HNSCC cell lines, usually coincident with the underexpression of p63.[16,44]

Differentiation and Mesenchymal Transition: NOTCH/TP63

Many of the expression profile studies in HNSCC contain a large number of genes that are thought to reflect the process of epithelial-to-mesenchymal transition (EMT), especially profiles of metastatic HNSCC. EMT is a biological process, wherein cells change from an epithelial phenotype to a mesenchymal-like phenotype. Because epithelial cells do not possess the cellular plasticity for metastatic dissemination, this process is a common occurrence in cancer cells.[4] *TP63* codes for p63, a p53-related transcription factor that, via its target genes, regulates differentiation in stratified epithelium, lineage specification, and subsequently proliferative potential. Mice lacking *TP63* undergo total failure of epidermal maturation.[45,46] In normally differentiated mature epithelium, *TP63* expression is present as a gradient. The highest level occurs in the basal epithelial cells, where it serves to antagonize *NOTCH1* expression. Rising superficially through the strata, *TP63* levels decrease and *NOTCH1* levels increase, driving terminal differentiation of the epithelial cell type (see Figure 33-1). In dysplastic mucosa, this patterning is lost, and *TP63* expression is evident throughout all layers of the epithelium. In addition, *TP63* overexpression and/or amplification are seen in the majority of HNSCC, and mutations were found in approximately 7% of tumors by exome sequencing.[15,16,47] An isoform of *TP63*, ΔNp63, known to contribute to cell survival, senescence suppression, and growth factor signaling, was also found to be specifically upregulated in HNSCC.[16,44]

Another recent finding that emerged from the HNSCC exome sequencing studies is the discovery of *NOTCH* family mutations in 15% to 20% of tumors, with most being present in *NOTCH1* (12% to 15%).[15,16] NOTCH signaling has been shown to influence cell survival, self-renewal capacity, and cell cycle exit, in addition to driving epithelial differentiation in concert with p63 and other signaling pathways. Ligands on adjacent cells bind to

the NOTCH receptor, resulting in the cleavage of intracellular portions of the receptor that subsequently translocate to the nucleus and drive the transcription of NOTCH target genes.[48] Overactivation of this pathway is believed to be tumorigenic in diffuse large B-cell lymphoma, T-cell acute lymphoblastic leukemia, and chronic lymphocytic leukemia. In those hematologic malignancies, translocations and activating mutations within NOTCH receptor genes have been observed.[49-51] In contrast, the observed *NOTCH* mutations in HNSCC are believed to be largely inactivating, loss-of-function–type mutations, suggesting a possible tumor suppressor role.[15,52] The exact role of NOTCH signaling in HNSCC remains to be elucidated and is likely tissue and/or context dependent, as has been observed in mouse models of epidermal and hematopoietic malignancies.[53,54]

Invasion and Metastasis: MMP/TGFβ-SMAD/NFκB/CSMD/VEGF

Like many cancers, HNSCC tumors metastasize primarily to the regional lymph nodes. The number of lymph node metastases in the neck, in more distant tissues, and the presence of extranodal spread are important prognostic factors, predictive of disseminated disease and survival. Although expression profile signatures of primary tumors that are predictive of metastasis have been identified, attempts to elucidate the mechanisms driving HNSCC metastasis are preliminary and in some cases conflicting.[4] Metastasis is a multifaceted process that ultimately results in a primary tumor "seeding" a distant anatomical site in the body. It involves several steps, one of which is invasion via the degradation of the extracellular matrix surrounding the primary tumor, in order to gain access to other areas of the body via the bloodstream or lymph system. Many studies have investigated the involvement of the matrix metalloproteinases (MMPs), which facilitate the degradation of the extracellular matrix. To date, strong associations have not been found, and treatments targeting MMPs have not achieved appreciable success in HNSCC.[55]

In the context of invasion, transforming growth factor β pathway (TGFβ), which normally functions to inhibit growth, has been implicated in HNSCC. TGFβ ligands bind to the receptors TGFBR1 and TGFBR2, resulting in phosphorylation of TGFBR1, which then activates the proteins SMAD2 and SMAD3. A SMAD complex is formed with the addition of SMAD4. This complex enters the nucleus and binds transcription factors, co-activators, and co-repressors, which modulate the expression of TGFβ target genes. Several of these are known to suppress cell proliferation, such as the cell cycle inhibitors cyclin-dependent kinase inhibitor 2. In addition, the TGFβ pathway has been identified as a key player in the EMT process.[56]

The TGFβ pathway has been implicated in HNSCC—most commonly through 18q deletion, which contains *SMAD2*, *SMAD3*, *SMAD4*, and the *TGFBRII* gene.[4] A recent mouse model found that conditional deletion of *SMAD4* in the head and neck epithelium was sufficient to generate invasive HNSCC. The loss of *SMAD4* expression in these animals correlated with increased expression of *TGFBRI* and increased activation of SMAD3, and the Fanconi anemia DNA repair pathway was found to be downregulated.[57] Previous studies reported missense mutations in *TGFBRII* in primary HNSCC, as well as mutations in *SMAD2* and *SMAD4* in some HNSCC cell lines.[58,59] The prevalence of such mutations, however, has been called into question by recent exome sequencing studies, which did not report any in the 106 tumors that were fully analyzed.[15,16] Recently, it has been demonstrated that reduced activity of the TGFβ pathway correlates with increased NF-κB signaling in HNSCC. Though alterations in TGFβ and NF-κB signaling have long been implicated in cancer, the exact mechanism(s) of their interaction, as well as their independent and/or cooperative contributions to invasion and metastasis in HNSCC, still need to be precisely elucidated.[60,61]

Tumors require blood vessels in order to grow to sizes larger than a few millimeters in diameter. These vessels facilitate nutrient and oxygen delivery, as well as metabolic by-product disposal. The exploitation of neo-angiogenesis, usually by producing angiogenic factors, is common to all solid tumors. Of the many inducers of angiogenesis, the strongest is vascular endothelial growth factor (VEGF). Many studies have linked VEGF expression to HNSCC prognosis, including a meta-analysis that found a significantly increased risk of 1.88, as well as an association with VEGF expression and metastasis to lymph nodes.[4] Although these data suggest a link between VEGF expression and outcome, neither VEGF nor EGFR expression has thus far been found to be a better prognostic indicator than HPV status.[62]

Both of the recent exome sequencing studies reported mutations in *CSMD3*, a putative adhesion factor that maps to 8p23 along with its family member, *CSMD1*. *CSMD1* is a putative tumor suppressor, the loss of which is associated with high tumor grade and poor prognosis in other cancers, and whose role in HNSCC remains under investigation.[15,16,63] Though functional studies have not yet been performed, these mutations may underlie a mechanism permitting the dissociation of cells from an otherwise cohesive sheet of cancerous epithelium, ultimately allowing for migration and metastasis of HNSCC tumors.

Future Directions

The elucidation of molecular pathways responsible for the pathogenesis of HNSCC has already led to the development and implementation of several targeted therapies.[25,30,35] Given the heterogeneity of this disease, future efforts will be required to identify effective targets in specific patient populations. One potential target may be miRNAs: several preliminary studies have identified a set of 66 commonly deregulated miRNAs in HNSCC that may in fact contribute to many of the cancerous phenotypes observed in this disease and are predictive of metastasis.[64,65] A recent study reports distinct miRNA expression profiles in HNSCC dependent on HPV status.[66] Similarly, epigenetic studies have identified promoter hypermethylation patterns that correspond to HPV status, as well as a series of changes that correspond to the development of cancer from a precancerous plaque.[67,68] The continuation of these early investigative efforts, in molecular markers of prognosis and treatment response, will be vital to our ability to generate tumor-tailored therapies for individual patients.

Key References

4. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011;11:9-22.
6. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363:24-35.
11. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354:567-578.
12. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med*. 2008;359:1116-1127.
15. Agrawal N, Frederick MJ, Pickering CR, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science*. 2011;333:1154-1157.
16. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science*. 2011;333:1157-1160.
17. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
32. Lu SL, Herrington H, Wang XJ. Mouse models for human head and neck squamous cell carcinomas. *Head Neck*. 2006;28:945-954.
35. Sharafinski ME, Ferris RL, Ferrone S, Grandis JR. Epidermal growth factor receptor targeted therapy of squamous cell carcinoma of the head and neck. *Head Neck*. 2010;32:1412-1421.
57. Bornstein S, White R, Malkoski S, et al. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. *J Clin Invest*. 2009;119:3408-3419.

References

1. Duvvuri U, Myers JN. Cancer of the head and neck is the sixth most common cancer worldwide. *Curr Probl Surg*. 2009;46:114-117.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.
3. American Cancer Society. *Cancer Facts and Figures 2012*. Surveillance Research; 2012. Retrieved from http://www.cancer.org/research/cancerfactsfigures/cancerfactsfigures/cancer-facts-figures-2012.
4. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011;11:9-22.
5. Human papillomavirus-associated cancers—United States, 2004-2008. *MMWR Morb Mortal Wkly Rep*. 2012;61:258-261.
6. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med*. 2010;363:24-35.
7. Cardesa A, Nadal A. Carcinoma of the head and neck in the HPV era. *Acta Dermatovenerol Alp Panonica Adriat*. 2011;20:161-173.
8. Park JW, Pitot HC, Strati K, et al. Deficiencies in the Fanconi anemia DNA damage response pathway increase sensitivity to HPV-associated head and neck cancer. *Cancer Res*. 2010;70:9959-9968.
9. Okano M, Gross TG. Acute or chronic life-threatening diseases associated with Epstein-Barr virus infection. *Am J Med Sci*. 2012;343:483-489.
10. Edge SBBD, Compton CC, et al. *AJCC Cancer Staging Manual*. 7th ed. New York, NY: Springer Verlag; 2009.
11. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006;354:567-578.
12. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med*. 2008;359:1116-1127.
13. Roesch-Ely M, Nees M, Karsai S, et al. Proteomic analysis reveals successive aberrations in protein expression from healthy mucosa to invasive head and neck cancer. *Oncogene*. 2007;26:54-64.
14. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol*. 2010;2:a001008.
15. Agrawal N, Frederick MJ, Pickering CR, et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science*. 2011;333:1154-1157.
16. Stransky N, Egloff AM, Tward AD, et al. The mutational landscape of head and neck squamous cell carcinoma. *Science*. 2011;333:1157-1160.
17. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
18. Poeta ML, Manola J, Goldwasser MA, et al. TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2007;357:2552-2561.
19. Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS. Dysregulated molecular networks in head and neck carcinogenesis. *Oral Oncol*. 2009;45:324-334.
20. Vousden KH, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol*. 2007;8:275-283.
21. Sailasree R, Abhilash A, Sathyan KM, Nalinakumari KR, Thomas S, Kannan S. Differential roles of p16INK4A and p14ARF genes in prognosis of oral carcinoma. *Cancer Epidemiol Biomarkers Prev*. 2008;17:414-420.
22. Pannone G, Rodolico V, Santoro A, et al. Evaluation of a combined triple method to detect causative HPV in oral and oropharyngeal squamous cell carcinomas: p16 immunohistochemistry, consensus PCR HPV-DNA, and in situ hybridization. *Infect Agent Cancer*. 2012;7:4.
23. Higuchi E, Oridate N, Homma A, et al. Prognostic significance of cyclin D1 and p16 in patients with intermediate-risk head and neck squamous cell carcinoma treated with docetaxel and concurrent radiotherapy. *Head Neck*. 2007;29:940-947.
24. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer*. 2011;11:558-572.
25. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. 2009;9:550-562.
26. Bian Y, Hall B, Sun ZJ, et al. Loss of TGF-β signaling and PTEN promotes head and neck squamous cell carcinoma through cellular senescence evasion and cancer-related inflammation. *Oncogene*. 2012;31:3322-3232.
27. Berger AH, Knudson AG, Pandolfi PP. A continuum model for tumour suppression. *Nature*. 2011;476:163-169.
28. Murugan AK, Hong NT, Fukui Y, Munirajan AK, Tsuchida N. Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas. *Int J Oncol*. 2008;32:101-111.
29. Henken FE, Banerjee NS, Snijders PJ, et al. PIK3CA-mediated PI3-kinase signalling is essential for HPV-induced transformation in vitro. *Mol Cancer*. 2011;10:71.
30. Kundu SK, Nestor M. Targeted therapy in head and neck cancer. *Tumour Biol*. 2012;33:707-721.
31. Castellano E, Downward J. RAS interaction with PI3K: more than just another effector pathway. *Genes Cancer*. 2011;2:261-274.
32. Lu SL, Herrington H, Wang XJ. Mouse models for human head and neck squamous cell carcinomas. *Head Neck*. 2006;28:945-954.
33. Narisawa-Saito M, Inagawa Y, Yoshimatsu Y, et al. A critical role of MYC for transformation of human cells by HPV16 E6E7 and oncogenic HRAS. *Carcinogenesis*. 2012;33:910-917.
34. Baines AT, Xu D, Der CJ. Inhibition of Ras for cancer treatment: the search continues. *Future Med Chem*. 2011;3:1787-1808.
35. Sharafinski ME, Ferris RL, Ferrone S, Grandis JR. Epidermal growth factor receptor targeted therapy of squamous cell carcinoma of the head and neck. *Head Neck*. 2010;32:1412-1421.
36. Hama T, Yuza Y, Saito Y, et al. Prognostic significance of epidermal growth factor receptor phosphorylation and mutation in head and neck squamous cell carcinoma. *Oncologist*. 2009;14:900-908.
37. Sok JC, Coppelli FM, Thomas SM, et al. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res*. 2006;12:5064-5073.
38. Szabo B, Nelhubel GA, Karpati A, et al. Clinical significance of genetic alterations and expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas. *Oral Oncol*. 2011;47:487-496.
39. Wheeler SE, Morariu EM, Bednash JS, et al. Lyn kinase mediates cell motility and tumor growth in EGFRvIII-expressing head and neck cancer. *Clin Cancer Res*. 2012;18:2850-2860.
40. Seiwert TY, Jagadeeswaran R, Faoro L, et al. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. *Cancer Res*. 2009;69:3021-3031.
41. Sequist LV, Waltman BA, Dias-Santagata D, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med*. 2011;3:75ra26.
42. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
43. Pacchiana G, Chiriaco C, Stella MC, et al. Monovalency unleashes the full therapeutic potential of the DN-30 anti-Met antibody. *J Biol Chem*. 2010;285:36149-36157.
44. Rocco JW, Leong CO, Kuperwasser N, DeYoung MP, Ellisen LW. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell*. *J*. 2006;9:45-56.
45. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature*. 1999;398:708-713.
46. Yang A, Schweitzer R, Sun D, et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature*. 1999;398:714-718.
47. Yugawa T, Narisawa-Saito M, Yoshimatsu Y, et al. DeltaNp63alpha repression of the Notch1 gene supports the proliferative capacity of normal human keratinocytes and cervical cancer cells. *Cancer Res*. 2010;70:4034-4044.
48. Dang TP. Notch, apoptosis and cancer. *Adv Exp Med Biol*. 2012;727:199-209.
49. Lee SY, Kumano K, Nakazaki K, et al. Gain-of-function mutations and copy number increases of Notch2 in diffuse large B-cell lymphoma. *Cancer Sci*. 2009;100:920-926.
50. Puente XS, Pinyol M, Quesada V, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 2011;475:101-105.
51. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306:269-271.
52. Dotto GP. Notch tumor suppressor function. *Oncogene*. 2008;27:5115-5123.

53. Nicolas M, Wolfer A, Raj K, et al. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet*. 2003;33:416-421.
54. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med*. 1996;183:2283-2291.
55. Rosenthal EL, Matrisian LM. Matrix metalloproteases in head and neck cancer. *Head Neck*. 2006;28:639-648.
56. Ikushima H, Miyazono K. TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer*. 2010;10:415-424.
57. Bornstein S, White R, Malkoski S, et al. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. *J Clin Invest*. 2009;119:3408-3419.
58. Qiu W, Schonleben F, Li X, Su GH. Disruption of transforming growth factor beta-Smad signaling pathway in head and neck squamous cell carcinoma as evidenced by mutations of SMAD2 and SMAD4. *Cancer Lett*. 2007;245:163-170.
59. Wang D, Song H, Evans JA, Lang JC, Schuller DE, Weghorst CM. Mutation and downregulation of the transforming growth factor beta type II receptor gene in primary squamous cell carcinomas of the head and neck. *Carcinogenesis*. 1997;18:2285-2290.
60. Cohen J, Chen Z, Lu SL, et al. Attenuated transforming growth factor beta signaling promotes nuclear factor-kappaB activation in head and neck cancer. *Cancer Res*. 2009;69:3415-3424.
61. Freudlsperger C, Bian Y, Contag Wise S, et al. TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. *Oncogene*. 2013;32:1549-1559.
62. Fei J, Hong A, Dobbins TA, et al. Prognostic significance of vascular endothelial growth factor in squamous cell carcinomas of the tonsil in relation to human papillomavirus status and epidermal growth factor receptor. *Ann Surg Oncol*. 2009;16:2908-2917.
63. Kamal M, Shaaban AM, Zhang L, et al. Loss of CSMD1 expression is associated with high tumour grade and poor survival in invasive ductal breast carcinoma. *Breast Cancer Res Treat*. 2010;121:555-563.
64. Babu JM, Prathibha R, Jijith VS, Hariharan R, Pillai MR. A miR-centric view of head and neck cancers. *Biochim Biophys Acta*. 2011;1816:67-72.
65. Chen Z, Jin Y, Yu D, et al. Down-regulation of the microRNA-99 family members in head and neck squamous cell carcinoma. *Oral Oncol*. 2012;48:686-691.
66. Lajer CB, Garnaes E, Friis-Hansen L, et al. The role of miRNAs in human papilloma virus (HPV)-associated cancers: bridging between HPV-related head and neck cancer and cervical cancer. *Br J Cancer*. 2012;106:1526-1534.
67. Worsham MJ, Stephen JK, Chen KM, et al. Delineating an epigenetic continuum in head and neck cancer. *Cancer Lett*. 2012. [Epub ahead of print].
68. Sartor MA, Dolinoy DC, Jones TR, et al. Genome-wide methylation and expression differences in HPV(+) and HPV(-) squamous cell carcinoma cell lines are consistent with divergent mechanisms of carcinogenesis. *Epigenetics*. 2011;6:777-787.
69. Graveland AP, Golusinski PJ, Buijze M, et al. Loss of heterozygosity at 9p and p53 immunopositivity in surgical margins predict local relapse in head and neck squamous cell carcinoma. *Int J Cancer*. 2011;128:1852-1859.
70. Schaaij-Visser TB, Graveland AP, Gauci S, et al. Differential proteomics identifies protein biomarkers that predict local relapse of head and neck squamous cell carcinomas. *Clin Cancer Res*. 2009;15:7666-7675.

Eric R. Fearon

34 Colon and Rectal Cancer

Colorectal cancer (CRC) development is a complex process. Causal agents and mechanisms include environmental and dietary factors and inherited and somatic mutations. Great progress has been made over the past 30 years toward defining the constellation of molecular alterations contributing to colorectal tumor development. Specific oncogene and tumor suppressor gene defects have been identified in tumors of various stages, with oncogenes being broadly defined as those genes affected by gain-of-function alterations in cancer, and tumor suppressor genes as the genes affected by loss-of-function alterations. Oncogenic activation of proto-oncogenes can result from many different mechanisms, including specific point mutations and rearrangements that alter gene structure and function, as well as from chromosomal rearrangements and gene amplifications that disrupt the regulated expression of the proto-oncogene. Tumor suppressor gene inactivation can also result from many different mechanisms, including localized mutations, such as nonsense or frameshift mutations, or complete loss of the gene. Besides mutational mechanisms, it is now clear that changes solely in the expression but not the structure or sequence of proto-oncogenes and tumor suppressor genes can lead to lead to oncogene activation or tumor suppressor gene inactivation. Although many proto-oncogenes and tumor suppressor genes encode proteins, they can also encode various noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). Thus the definitions of *oncogene* or *tumor suppressor gene* are essentially agnostic with respect to the function of the gene in the cell, but instead refer to whether the resultant genetic or epigenetic defect in a given gene leads to increased or novel gene function (i.e., oncogene) or loss of gene function (i.e., tumor suppressor gene). Finally, although the vast majority of gene defects found in colorectal cancer are somatic, inherited mutations in selected tumor suppressor genes have a critical role in a number of CRC predisposition syndromes.

The principal objectives of this chapter are to review the following topics: (1) the epidemiology of CRC; (2) the sequence of histopathologic alterations in the course of the progression to malignancy; (3) the genetic basis of various inherited CRC syndromes and the relevance of certain inherited syndromes to sporadic CRC development; (4) somatic oncogene and tumor suppressor gene defects and epigenetic changes in CRC; (5) some of the apparent variations in gene defects associated with different precursor lesions at risk of progressing to CRC; and (6) the potential clinical utility of the genetic alterations in early detection and clinical management of patients and families affected by CRC.

Epidemiology

CRC is the second leading cause of cancer deaths in the United States.[1] In 2013, it is anticipated that nearly 143,000 individuals in the United States will be diagnosed with colon or rectal cancer, and about 51,000 will die from the disease.[1] Male and female incidence and survival are very similar.[1] Important differences in prognosis have been observed in different racial groups in the United States.[1] These differences may reflect a combination of factors, including differences in access and adherence to screening tests, tumor location and size at diagnosis, and appropriateness of treatment.[2,3] At this point, there is less evidence that differences in molecular alterations among tumors (intertumoral genetic and epigenetic heterogeneity) have a major contributing role in accounting for differences in prognosis among racial groups, though further work is needed.[3]

Most cases of CRC are considered to be sporadic, indicating that clear-cut familial or genetic predisposition factors are not readily apparent. However, based in part on family studies (i.e., twin and kindred studies), it is estimated that more than 25% to 30% of CRC cases may have a hereditary component.[4,5] Less than a fourth of this collection of cases with a presumptive hereditary component (i.e., less than 5% of all cases) occur in a setting with a family history and/or clinical features indicative of a highly penetrant, single-gene-mutation cancer syndrome predisposing

to CRC development.[4,5] Gene alterations or DNA sequence variations with key contributing roles in most familial cases remain to be defined. The familial cases may be a heterogeneous group, in which modest to moderate predisposition to CRC is possibly conferred by an undetermined number of potentially common or rare genetic variations.[4]

In addition to family history, other risk factors for CRC include older age, chronic inflammatory bowel disease, heavy alcohol use, and diets rich in unsaturated fats and red meat and refined starches and low in fruits and vegetables.[6,7] Evidence for a protective effect against CRC for aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) as well as hormone supplementation in postmenopausal women has been offered, albeit with attendant risks for these agents.[6] An unfortunate reality is that many of the key dietary, lifestyle, and environmental factors contributing to CRC in the United States and other Western countries are uncertain, and the majority of incident CRCs arise in people with no well-defined risk factor. On a more optimistic note, a large fraction of CRC deaths seem to be preventable by early detection. By some estimates, improved adoption and implementation of current screening recommendations for colorectal cancer could save thousands of lives per year.[8]

Histopathologic Changes in Colorectal Carcinogenesis: The Adenoma-Carcinoma Sequence

The surface of the large intestine is covered by an epithelium, characterized by finger-like invaginations into the underlying stroma; the invaginations are termed *crypts*. Stem cells near the base of each crypt give rise to distinct cell types required for the intestine's resorptive, secretory, and endocrine functions. During their differentiation, epithelial cells migrate from the base of the crypt to the surface, and the cells are ultimately shed into the gut lumen. With the exception of the stem cells, the colon epithelium is turned over within a few days.

Among the different types of benign gastrointestinal lesions, a generic term for any localized lesion protruding above the surrounding mucosal surface is *polyp*. Many polyps, particularly small polyps 5 mm or less in size, are of the hyperplastic type, with characteristic serrated glands and distended goblet (mucus-producing) cells. However, although the role of certain serrated polyp lesions other than hyperplastic polyps in CRC development is discussed later, the adenomatous polyp or adenoma is believed to be the precursor lesion to the vast majority of CRCs.[9,10] Both gross and histopathologic features can be used to distinguish adenomas. Grossly, the size is measured and the morphology can be described as pedunculated (with a stalk), sessile (flat), or semisessile. Among the histopathologic features, the degree of dysplasia and glandular architecture are used to distinguish lesions, and these features may have value for predicting the likelihood that a lesion contains a focus of cancer or its risk of progression to cancer.

The proposal that many CRCs arise from adenomatous lesions is supported by at least three lines of evidence. First, a few longitudinal studies have assessed the risk of subsequent CRC development in individuals with adenomatous polyps.[11] In these studies it became apparent that patients who did not have their adenomas removed had an approximately eightfold increased risk of CRC compared with the group that had their adenomas removed. Notably, after polypectomy, patients did not show a clear increase in CRC incidence in comparison with a control group without adenomas, suggesting that adenomatous polyp removal had a therapeutic effect.[11] Second, histopathologic studies have shown that foci of carcinoma can often be detected in adenomatous polyps, particularly those with increased size, dysplasia, and villous histopathology. Third, individuals affected by syndromes that strongly predispose to the development of hundreds of adenomas, such as familial adenomatous polyposis (discussed later), invariably develop colorectal cancers by the third to fifth decades of life, if their colons are not removed.

Although adenomatous polyps as a group are well established to have a risk of progression to cancer,[12] some studies have suggested that patients who develop hyperplastic polyps may have an increased risk of adenomas.[13,14] Also, patients with numerous hyperplastic polyps (e.g., patients with juvenile hyperplastic polyposis syndrome) have a clearly increased risk of CRC. As suggested previously and as discussed further later, it has been increasingly appreciated that certain polypoid lesions—such as "sessile serrated adenomas," which share some serrated morphologic features with hyperplastic polyps—do indeed show an increased cancer risk.[9,10] Most carcinomas arising from sessile serrated adenomas seem to be associated with distinct molecular defects that are discussed later (e.g., CpG island methylation phenotype, CIMP).

At least two other pathways leading to CRC and that are not associated with overt development of adenomatous polyps as precursors can be noted: chronic inflammatory bowel diseases (particularly ulcerative colitis [UC] and to a lesser extent Crohn disease) and flat adenoma syndromes. UC is a chronic inflammatory disease of largely unknown etiology. The possible precursor lesions to cancer in patients with UC include dysplasia and flat adenomatous plaques.[15] In a subset of patients with hereditary cancer syndromes, as well as some sporadic cases, colon cancer seems to develop directly from flat adenoma or intraepithelial dysplasia.[16] In these cases the progression from benign lesion to overt

Table 34-1 Genetics of Inherited Colorectal Tumor Syndromes

Syndrome	Features Commonly Seen in Affected Individuals	Gene Defect
Familial adenomatous polyposis (FAP)	Multiple adenomatous polyps (>100) and carcinomas of the colon and rectum; duodenal polyps and carcinomas; fundic gland polyps in the stomach; congenital hypertrophy of retinal pigment epithelium (CHRPE)	*APC*
Gardner syndrome	Same as FAP; also desmoid tumors and mandibular osteomas	*APC*
Turcot syndrome	Polyposis and colorectal cancer with brain tumors (medulloblastoma)	*APC*
Colorectal cancer and brain tumors	Colorectal cancer without polyposis and brain tumors (glioblastoma)	*MLH1, PMS2*
Attenuated adenomatous polyposis coli (AAPC)	Fewer than 100 polyps, though marked variation in polyp number (from 5 to >1000 polyps) is seen in mutation carriers within a single family	*APC* (predominantly 5′ mutations)
Lynch syndrome (hereditary nonpolyposis colorectal cancer [HNPCC])	Colorectal cancer without polyposis; other cancers include endometrial, ovarian, stomach cancer; urothelial, brain	*MSH2, MLH, PMS2, MSH6*
Peutz-Jeghers syndrome	Hamartomatous polyps throughout the gastrointestinal tract; mucocutaneous pigmentation; estimated 9-to 13-fold increased risk of gastrointestinal (GI) and non-GI cancers	*LKB1/STK11*
Cowden disease	Multiple hamartomas involving breast, thyroid, skin, central nervous system (CNS), and GI tract; increased risk of breast, uterus, and thyroid cancer; risk of GI cancer unclear	*PTEN*
Juvenile polyposis syndrome	Multiple hamartomatous/juvenile polyps with predominance in colon and stomach; variable increase in colorectal and stomach cancer risk; facial changes	*DPC4* *BMPR1A* *PTEN*
MYH-associated polyposis (MAP)	Multiple adenomatous gastrointestinal polyps, autosomal recessive often associated with somatic K-Ras mutations	*MYH*
Multiple adenoma and colorectal cancer	Multiple colorectal adenomas and colorectal cancer; endometrial cancer and brain tumors in some individuals and families	*POLD1, POLE*
Hereditary mixed polyp syndrome	Multiple types of colorectal polyps (e.g., Peutz-Jeghers polyps, juvenile polyps, serrated lesions, conventional adenomas) and colorectal cancer	*GREM1*

carcinoma may be more rapid than in the normal adenoma-carcinoma progression.

Hereditary Colorectal Cancer Syndromes and Molecular Pathways of Colorectal Carcinogenesis

It is now generally accepted that the accumulation of somatic mutations and epigenetic defects drives the initiation and progression of tumor development. The constellation of somatic mutations that commonly accumulate in a clonal fashion in CRCs has largely been defined.[17-20] However, only a limited subset of this collection of gene defects is usually present in any individual tumor. Thus far, the somatic mutations of greatest initial interest to the field have been those that are recurrent and clonal (i.e., present in all, or nearly all, neoplastic cells of a primary tumor at a given stage, but not present in the normal cells of the patient). It is inferred that such recurrent, clonal mutations are causal in promoting further tumor outgrowth/progression, because somatic mutations can only become clonal by a limited number of mechanisms. The genetic alteration itself could have been selected for because it provided the cell with a growth advantage, allowing it to become the predominant cell type in the tumor (clonal expansion). Alternatively, the specific alteration detected might have arisen coincident with another, perhaps undetected, alteration that was the crucial change underlying clonal outgrowth.

As noted previously, only about 5% of all CRC cases are associated with defined highly penetrant cancer syndromes. The bulk of these cases are attributable to hereditary nonpolyposis colorectal cancer syndromes (HNPCCs), with another significant subset associated with the familial adenomatous polyposis (FAP) syndrome (Table 34-1). A few other syndromes comprise the remaining highly penetrant CRC syndrome cases.

Adenomatous polyposis coli Gene: Gatekeeper in Familial Adenomatous Polyposis and Sporadic Cancer

Familial Adenomatous Polyposis

FAP is an autosomal dominant syndrome affecting about 1 in 8000 individuals in the United States and accounting for about 0.5% of CRCs.[4,5] Hundreds to thousands of adenomas

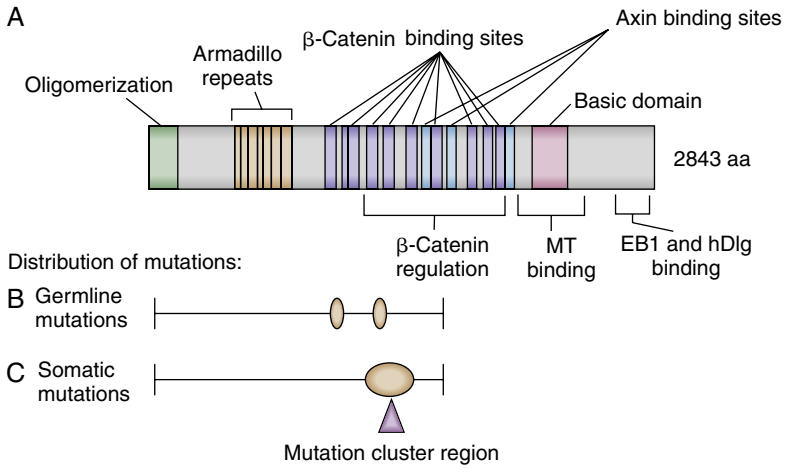


Figure 34-1 Schematic representation of the adenomatous polyposis coli (APC) protein and mutation histograms **(A)** Selected sequence motifs of the 2943-amino-acid APC protein and its interaction partners are indicated. The amino-terminal region has a domain that regulates its oligomerization. Repeated sequences with similarity to the *Drosophila* armadillo protein and its vertebrate homologue β-catenin (so-called armadillo repeats) are localized in the amino-terminal third of APC. Multiple independent 20-amino-acid repeats mediating binding to β-catenin and several binding sites for the Axin protein (termed *SAMP repeats*) are localized in the central third of APC. The carboxy-terminal third contains a basic region that is involved in microtubule (MT) binding and mediates interactions with the proteins EB1 and hDlg. **(B)** The frequency and distribution of germline mutations in familial adenomatous polyposis (FAP) patients are indicated with respect to the *APC* coding region. Virtually all mutations result in premature truncation at or very close to the mutation position. Two apparent mutational hotspots are seen at codons 1061 and 1309. **(C)** Frequency and distribution of somatic *APC* mutations in sporadic colorectal cancers are indicated. The mutations appear to predominate in the "mutation cluster region" (MCR), and mutations at codons 1309 and 1450 are most common. ***(A-C** with permission from Polakis P. Mutations in the APC gene and their implications for protein structure and function.* Curr Opin Genet Develop *1995;5:66).*

arise in the large bowel and rectum beginning in the second decade of life. Although only a fraction of the adenomas may progress to cancer, the lifetime incidence of colorectal cancer in untreated FAP patients approaches 100% with a mean age at diagnosis of 39 years, necessitating the prophylactic removal of the patient's colon early in adult life.[4] The gene that when mutated underlies FAP is the *adenomatous polyposis coli* (*APC*) tumor suppressor gene on chromosome 5q21. Germline mutations have been identified in one *APC* allele of affected individuals in 90% to 95% of families with FAP studied. More than 95% of mutations lead to premature truncation of APC protein synthesis—about two thirds by small insertions or deletions leading to a frameshift, and the remainder by introducing a stop codon[21] (Figure 34-1). Up to 25% of FAP cases seem to be caused by de novo germline mutations and therefore do not show the characteristic autosomal pattern of inheritance.[4] Moreover, about 20% of individuals with a de novo *APC* mutation display somatic mosaicism for the mutation in their tissues, with the mutant *APC* allele present in only subsets of cells within a given tissue and/or in germ cells.[4] Some of these cases with somatic mosaicism may manifest milder polyposis phenotypes than individuals carrying inherited germline *APC* mutations, as the defective *APC* allele is present in only a fraction of cells in the somatic mosaicism situation.

Several extracolonic tumors and symptoms are associated with FAP (see Table 34-1). The combination of polyposis with brain tumors (in particular, medulloblastoma in pediatric cases) has been termed *Turcot syndrome*.[22] Gardner syndrome comprises extensive polyposis with epidermoid cysts, desmoid tumors, and osteomas and seems to be correlated with mutations between *APC* codons 1403 and 1578.[23,24] Upper gastrointestinal polyps are responsible for a large proportion of morbidity and mortality in FAP patients after prophylactic total colectomy. This is particularly true for the duodenal cancers that develop in 4% to 12% of patients.[4,25,26] Benign gastric fundic gland polyps and gastric adenomas, potential premalignant precursor lesions of gastric cancer, are observed with increased frequency, ultimately leading to stomach cancer in 0.5% of cases.[4,25,26] In only a small percentage of FAP cases, thyroid cancer, bile duct cancer, hepatoblastoma (pediatric), and central nervous system tumors such as medulloblastoma are observed. In addition to FAP, rare germline *APC* variants appear to play a role in other familial CRC cases.[27] Epidemiologic studies have revealed that the *APC* I1307K (isoleucine-to-lysine substitution at codon 1307) allele is present only in individuals of Ashkenazi Jewish origin, and those who carried the I1307K allele had a roughly twofold increased risk of developing CRC.[27] The mechanism underlying increased risk of adenomas and CRC in carriers of the *APC* I1307K allele is that the allele appears to be more susceptible than the wild-type APC allele to somatic mutations—particularly insertion and deletion of one or a few nucleotides near the homopolymeric adenine tract created by the lysine 1307 codon substitution, resulting in frameshift mutations.[27]

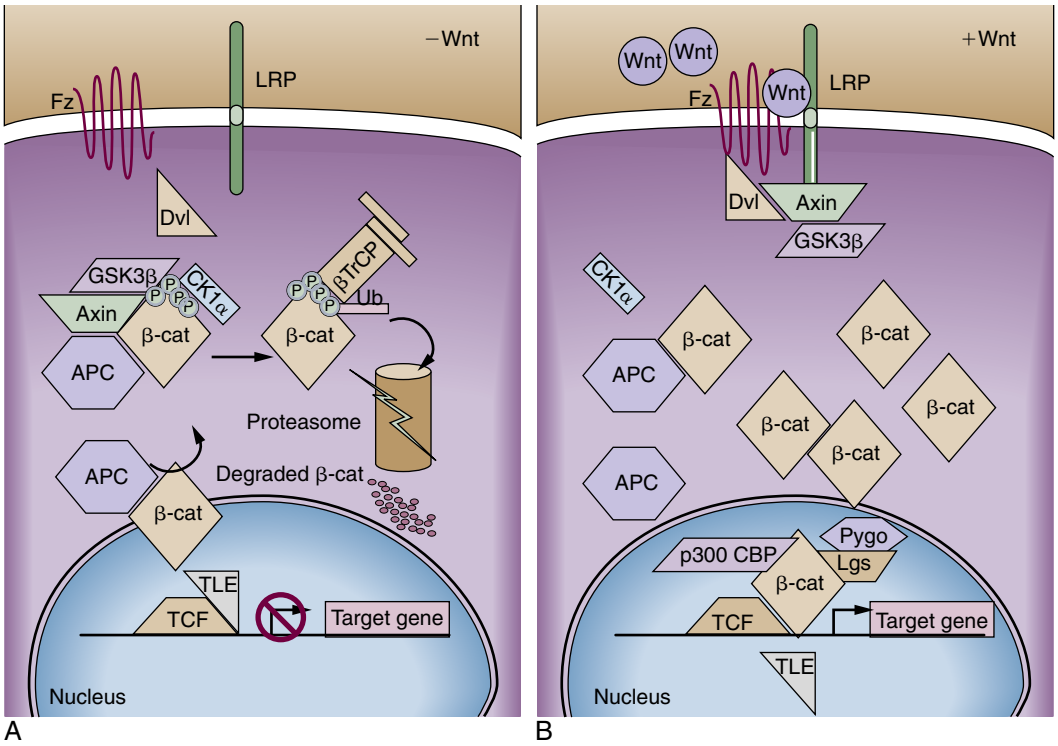


Figure 34-2 Model of adenomatous polyposis coli (APC) and β-catenin function **(A)** The APC protein is part of a "destruction complex" containing glycogen synthase kinase 3β (GSK3β), Axin, and casein kinase 1α (CK1α), which phosphorylates β-catenin in conserved serine and threonine residues in the N terminus. This phosphorylation creates an epitope for recognition by the F-box protein β-TrCP as part of a ubiquitin ligase complex, which leads to polyubiquitination and proteasomal degradation of β-catenin. Hence, in most cells free β-catenin levels are kept low and transcription of target genes is repressed by recruitment of repressors of the transducin-like enhancer of split family (TLE). **(B)** On binding of Wnt ligands to a cognate receptor complex consisting of one member of the Frizzled (Fz) family and one member of the low-density-lipoprotein receptor-related protein family (i.e., LRP5 or LRP6), the destruction complex is disassembled in part by recruitment of Axin to the LRP receptors and in part by not clearly defined action of disheveled proteins (Dvls). Similarly inactivating mutations of the APC protein or the Axin1 protein or mutations of the conserved phosphorylation sites of β-catenin lead to the accumulation of free β-catenin, which enters the nucleus and activates transcription of target genes by displacement of co-repressors and recruitment of coactivators such as CBP/p300 and a complex of the homologues of *Drosophila* Legless (Lgs) and Pygopus (Pygo).

Somatic *APC* Mutations in Sporadic Tumors

Notwithstanding the critical role of the *APC* gene in FAP and related variant syndromes, the *APC* gene has an even more prominent role in sporadic colorectal tumors, as about 80% of sporadic colorectal adenomas and carcinomas have somatic mutations inactivating *APC*,[28] the overwhelming majority of which lead to premature truncation of the APC protein. *APC* mutations have been found in a number of the earliest sporadic lesions analyzed, including microscopic adenomas composed of only a few dysplastic glands.[29] As predicted by Knudson's two-hit model for tumor suppressor genes, both *APC* alleles appear to be inactivated in the majority of colorectal adenomas and carcinoma, as a result of localized mutations in both alleles or localized mutation in one allele and chromosomal loss of the remaining allele.[28]

APC Protein Function

The APC tumor suppressor gene encodes a large protein of approximately 300 kDa (see Figure 34-1). The APC protein likely has multiple functions in the cell,[30] but the best understood function of APC is as a major binding partner and regulator of the β-catenin protein.[31] β-Catenin was first identified because of its role in linking the cytoplasmic domain of the E-cadherin cell-cell adhesion molecule to the cortical actin cytoskeleton, via binding to the adaptor molecule α-catenin. Based on studies from many different investigators, a model has been developed to explain the biologic significance of APC's interaction with β-catenin.[31] The model proposes that in the absence of an activating Wnt ligand signal, APC binds to and collaborates with the scaffold protein Axin to promote sequential phosphorylation by casein kinase I and glycogen synthase kinase-3β (GSK3β) of several conserved serine/threonine residues in the N-terminal region of β-catenin, thereby targeting β-catenin for ubiquitination and proteasomal degradation (Figure 34-2). In a physiologic setting, the activating Wnt ligands inhibit degradation of β-catenin via binding to their cognate receptor complex of a Frizzled and an LRP5/6 protein.

In the roughly 80% of CRCs where both *APC* alleles are defective, the coordinated destruction of β-catenin is

severely impaired, essentially mimicking constitutive activation of Wnt ligand-mediated signaling (see Figure 34-2). As a result, β-catenin accumulates in the cytoplasm, complexes with DNA binding proteins of the TCF (T-cell factor family)/Lef (lymphoid enhancer family) family, and translocates to the nucleus. Once there, β-catenin functions as a transcriptional co-activator, activating the expression of TCF-regulated genes. In a subset of the cancers that lack mutations in APC, somatic mutations in β-catenin have been found.[32] A critical net consequence of β-catenin stabilization by APC inactivation or other somatic mutations in CRCs is that the constitutive activation of β-catenin/TCF transcription appears to promote a stem or progenitor cell phenotype in the affected epithelial cells independent of their position in the crypt compartment.[31] Normally, β-catenin/TCF activation is restricted to the crypt base, especially in the so-called crypt base columnar stem cells that are characterized by expression of the Wnt-regulated Lgr5 gene and protein.[31]

Further work has implicated β-catenin stabilization not only in the establishment of a crypt progenitor program but also in the spatial organization and migratory pattern of the cells in the continuous renewal of crypt.[31] Strikingly, feedback inhibitors functioning in the Wnt/β-catenin/TCF pathway are prominent among the critical proteins encoded by β-catenin/TCF-regulated genes, such as the AXIN2, DKK1, NKD1, APCDD1, and WIF-1 proteins, all of which function in some fashion to antagonize Wnt signaling.[31] In CRCs with *APC* mutations, the ability of the induced feedback regulator proteins to interfere with stabilized β-catenin is largely or entirely abrogated, because the induced Wnt-pathway feedback regulators function upstream of or at the level of the APC protein in the pathway.[31] Interestingly, some of the induced Wnt pathway feedback regulators that are highly expressed in CRCs with *APC* mutations, such as AXIN2, may play important contributing roles in CRC progression, such as through the ability of AXIN2 to interact with other proteins and pathways in the promotion of epithelial-mesenchymal transition and invasive phenotypes.[33]

Mouse Models of FAP and Genetic and Epigenetic Modifiers

An important mouse genetic model of intestinal tumorigenesis known as the *Min* (for *multiple intestinal neoplasia*) mouse was described more than two decades ago.[34] The *Min* mouse carries a germline mutation in the murine homologue of the *APC* gene, resulting in truncation of the murine Apc protein at codon 850. The intestinal tumor phenotype of *Min* or *Apc(Min)* mice is similar, but not identical, to that seen in FAP patients, with *Apc(Min)* mice developing largely small intestinal tumors, in contrast to the colorectal lesions seen in FAP patients.

Other cellular genes can significantly influence the number of polyps that arise in mice that are heterozygous for the *Apc(Min)* mutant allele or other mutant Apc alleles. For instance, when the Apc*(Min)* mutation was introduced into mice of varying genetic backgrounds, significant variability in the number of intestinal tumors was seen. This finding was attributable to the effects of strain-specific modifier genes unlinked to the *Apc* locus, particularly the modifier gene Pla2g2a, which encodes a secreted phospholipase A2 protein.[35] Other genes that substantially modify intestinal tumorigenesis in *Apc*-mutant mice include genes encoding DNA methyltransferases. Specifically, mice that carry the *Apc(Min)* mutation and a germline defect in one allele of the maintenance DNA methyltransferase *Dnmt1* or a gut-specific deletion of both *Dnmt3* alleles have a two- to threefold reduction in macroscopic polyp number.[36,37] Treatment of *Apc(Min)* mice with 5-azacytidine, a pharmacologic inhibitor of DNA methyltransferases, resulted in a more than fivefold reduction in polyp number.[36] Combining the genetic and pharmacologic manipulations of DNA methyltransferase activity synergistically reduced the polyp number roughly 50-fold.[36] Treatment of mice with NSAIDs that inhibit COX-1 and COX-2 or agents that specifically inhibit COX-2 activity also markedly inhibited adenoma formation.[38,39] The findings highlight the utility of the mouse genetic models for identifying novel genes and pharmacologic agents that modify intestinal tumor development.

Other Forms of Intestinal Polyposis

Some patients with germline mutations in the *APC* gene have a milder or attenuated FAP (AFAP) phenotype, due to the nature of the germline *APC* mutation that they carry and/or other genetic variants that they may carry that modify the severity of their polyposis phenotype.[4,5] However, many of the individuals who have an AFAP phenotype and typically present to clinical attention between 40 and 60 years of age with fewer than a dozen to a hundred polyps do not carry a germline *APC* mutation.[4,5] Rather, these affected individuals carry homozygous mutations in the *MYH* gene encoding the base-excision repair pathway protein MutYH. The syndrome is often termed *MAP* for *MYH*-associated polyposis syndrome.[4,5] The mutations in the *MYH* gene lead to constitutional defects in the repair of oxidative DNA damage in cells, with increased GC-to-AT base-pair transversions arising. As a result of the increased mutation frequency, in the colon, somatic mutations in various genes likely arise at increased frequency, with the somatic mutations contributing to adenoma formation.

Some individuals and families who develop 10 to 100 or more adenomas during adult life, and who also develop CRC at an increased rate unless managed clinically, have been found to lack germline mutations in the *APC* or *MYH*

genes. Recent studies have shown that some of these affected individuals and families carry heterozygous germline mutations in the *POLD1* and *POLE* genes encoding DNA polymerases δ and ε, respectively.[40] The mutations appear to affect the proofreading domains of the polymerases. In the case of *POLD1* mutations, affected individuals appear to be predisposed not only to adenomas and CRC but also to endometrial cancer and perhaps brain tumors.[40] The colon tumors arising in *POLD1* and *POLE* mutation carriers were found to have inactivated the remaining wild-type copy of the gene by loss of heterozygosity (LOH). As a result, the tumors lack intact polymerases δ or ε function, depending on the nature of the patient's germline mutation, and the tumors manifest a markedly greater frequency of base-substitution mutations relative to similar stage colon tumors with intact polymerase δ and ε function, presumably as a direct result of the deficits in polymerase δ or ε function.[40]

Other intestinal polyposis syndromes in which patients manifest numerous nonadenomatous lesions have also been described.[4,5] Several of these syndromes have an increased risk of gastrointestinal and/or non-gastrointestinal cancers (see Table 34-1). Starting from childhood, patients with the autosomal dominant juvenile polyposis syndrome (JPS) develop multiple hamartomatous polyps throughout the gastrointestinal tract with some preference for the colon and the stomach. JPS is a genetically heterogeneous disease, with inactivating mutations in the SMAD4 and BMPR1A genes among the underlying genetic bases. Cowden syndrome is an autosomal dominant syndrome in which affected individuals develop macrocephaly and hamartomas in many organ sites, including the breast, thyroid, skin, central nervous system, and gastrointestinal tract.[4,5] The gene responsible for Cowden syndrome is the *PTEN* tumor suppressor gene on chromosome 10q23. Acting as a phospholipid-phosphatase, the PTEN protein is a major antagonist of the phosphatidylinositol-3-kinase (PI3K) survival pathway. In a small number of nonfamilial cases of juvenile polyposis lacking other features of Cowden syndrome, *PTEN* germline mutations have been reported.[4,5] Peutz-Jeghers syndrome, a rare autosomal dominant condition affecting fewer than 1 in 25,000, is characterized by gastrointestinal hamartomatous polyps and mucocutaneous melanin deposition.[4,5] The hamartomatous polyps contain essentially normal epithelial cells, but the mucosal components are arranged abnormally. Germline mutations in the *LKB1/STK11* tumor suppressor gene on chromosome 19p can be seen in a significant fraction of cases.[4,5] Inactivation of this tumor suppressor gene leads to the hyperactivation of the mammalian target of rapamycin (mTOR) pathway, which is responsible for integrating the nutritional supply with cell proliferation and growth. Finally, recent studies of a rare inherited syndrome known as hereditary mixed polyposis syndrome (HMPS), in which individuals are predisposed to develop a range of lesions, including Peutz-Jeghers polyps, juvenile polyps, serrated lesions, conventional adenomas, and CRC, is due in some families to germline mutations that lead to overexpression of the bone morphogenetic protein (BMP) antagonist GREM1.[41] Thus, although HMPS is connected to JPS, based on the fact that BMPs signal through the transforming growth factor β (TGF-β) pathway, further work is needed to understand how derangements in TGF-β and BMP signaling contribute to predisposition to colorectal tumors.

DNA Mismatch Repair Deficiency and Lynch Syndrome

Hereditary nonpolyposis colorectal cancer (HNPCC) was arguably the first inherited cancer syndrome to be well described in the literature. In 1913, Warthin presented a particularly striking example of a three-generation family with CRC and other cancers, including gastric cancer and tumors of the female reproductive tract.[42] Following Warthin's lead, Lynch and others described kindreds with autosomal dominant patterns of CRC, not accompanied by extensive polyposis.[43] In such families, CRCs of early onset were seen, often along with cancers in some other organs including gastric, uterine endometrial, ovarian, small bowel, renal, and hepatobiliary cancers.[44] In recognition of Dr. Henry Lynch's major contributions to the field, HNPCC is often referred to as *Lynch syndrome*.

Criteria for the diagnosis of Lynch syndrome in families have often included the following: affected families must show Lynch syndrome–typical tumors in three relatives (one being a first-degree relative to the other two) in at least two successive generations, with one of the tumors occurring before age 50 (so-called 3-2-1 rule). The sensitivity of these diagnostic criteria is considerably less than 100% for identifying those affected by Lynch syndrome, and the specificity of the criteria is also an issue.[4] However, the diagnostic criteria proved critical for the initial discovery of the germline mutations underlying Lynch syndrome, and the criteria have also been useful in clinical assessment over the past 15 to 20 years since the genetic bases of Lynch syndrome were uncovered. By way of a brief background, the early genetic work indicated genetic heterogeneity for Lynch syndrome, with mapping of the predisposing gene to chromosome 2p in some families[45] and to chromosome 3p in other families.[46] In yet other families with Lynch syndrome, no evidence for linkage to chromosome 2p or 3p was found.

To explore the relevance of Knudson's two-hit hypothesis for Lynch syndrome genes, investigators initially sought to demonstrate loss of parental heterozygosity for chromosome 2p in cancers from individuals carrying a defect in

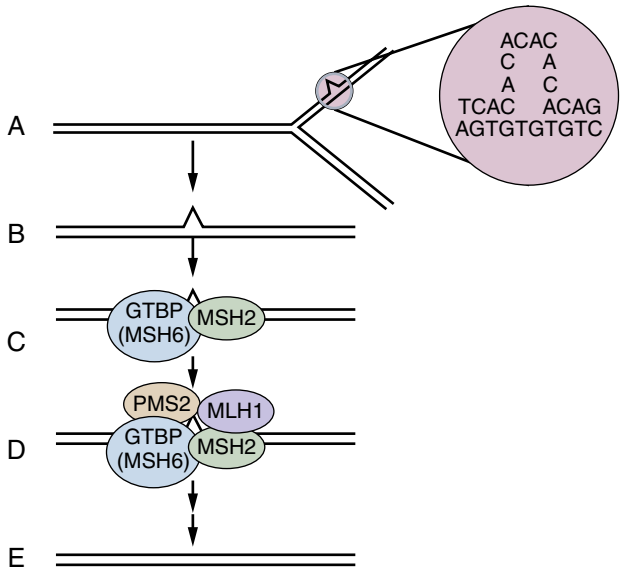


Figure 34-3 Mismatch repair pathway in human cells (A, B) During DNA replication, DNA mismatches may arise, such as from strand slippage (shown) or misincorporation of bases (not shown). **(C)** The mismatch is recognized by MutS homologues, perhaps most often MSH2 and GTBP/MSH6, although MSH5 may substitute for GTBP/MSH6 in some cases. **(D, E)** MutL homologues, such as MLH1 and PMS2, are recruited to the complex, and the mismatch is repaired through the action of a number of proteins, including an exonuclease, helicase, DNA polymerase, and ligase. ***A-E*** *with permission from Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer.* Cell. *1996;87:159-170.*

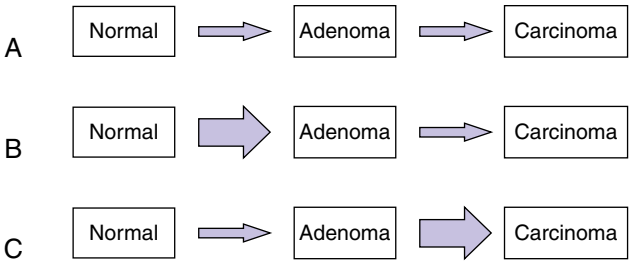


Figure 34-4 Relative effects of germline mutations on tumor initiation and progression (A) In sporadic cancers, both initiation of a neoplastic lesion (e.g., the adenoma) and progression to an advanced lesion (i.e., the carcinoma) are rate-limiting events because two somatic mutations are required for inactivation of tumor suppressors such as adenomatous polyposis coli (APC) (initiation of adenoma) and p53 (tumor progression). **(B)** In the case of familial adenomatous polyposis (FAP), germline inactivation of one APC allele markedly increases the formation of adenomas, because inactivation of both APC alleles is a critical (rate-limiting) event in adenoma formation, and those with inactivation of one allele in all colonic cells need only inactivate the remaining APC allele to initiate adenoma formation. **(C)** In the case of Lynch syndrome (hereditary nonpolyposis colorectal cancer [HNPCC]), germline inactivation of one of the mismatch-repair genes (e.g., *MSH2* or *MLH1*) coupled with somatic inactivation of the remaining allele in an initiated lesion (e.g., an early adenoma) greatly increases the mutation rate, and subsequently the rate and speed of progression to more advanced lesions. *With permission from Bettingon et al. The serrated pathway to colorectal cancer: current concepts and challenges. Histopathology. 2013; 62:367-386.*

that particular predisposition gene.[45] However, not only was there no LOH of chromosome 2p sequences in the cancers, but microsatellite DNA sequences examined in the analyses demonstrated marked length variations in tumor tissues compared with the patient's normal tissue. Microsatellite changes were seen at many different loci scattered throughout the genome and in all tumors from patients with Lynch syndrome.[45] The phenotype was termed the *microsatellite instability*" (MSI) or *replication error* (RER) phenotype. Cancers with evidence of microsatellite instability at more than 40% of a panel of mononucleotide and dinucleotide sequences are termed *high-frequency MSI* (MSI-H) cancers. Although most CRCs display no instability when a panel of microsatellite tracts are studied—so-called microsatellite stable (MSS) cases—a subset of cancers show low-frequency instability of the microsatellite markers, termed *MSI-L*.

The finding of MSI-H in the cancers was noteworthy because similar DNA instability phenotypes had previously been seen in mutant yeast strains with defective DNA mismatch repair genes. The prediction that defects in one or more DNA mismatch repair genes might underlie Lynch syndrome was quickly borne out (Figure 34-3). A human homologue of the bacterial *mutS* mismatch repair gene, designated *MSH2*, was mapped to chromosome 2p, and one allele was found to be mutated in the germline of a subset of Lynch syndrome patients, with the remaining allele inactivated in cancers arising in the mutation carriers. Other genes involved in DNA mismatch repair were studied and found mutated in other groups of Lynch syndrome patients, including the *MLH1* gene on chromosome 3p, the *PMS2* gene on 7q, and the *GTBP/MSH6* gene on chromosome 2p.[47] Mutations in the *MSH2* and *MLH1* genes are a far more common cause of Lynch syndrome than mutations in the other mismatch repair genes, with *MSH2* and *MLH1* mutations together accounting for about 70% to 75% of the known mutations in Lynch syndrome patients.[47]

In the normal cells of a patient with Lynch syndrome, DNA repair is rarely impaired, because the cells have a wild-type copy of the gene (i.e., the copy inherited from the nonaffected parent). However, during cancer development, the remaining wild-type allele is inactivated by a somatic mutation. Following inactivation of the wild-type allele, full mismatch repair activity is lost (see Figure 34-3). Then, affected cells manifest a mutator phenotype and accumulate mutations in a much more rapid fashion. Lynch syndrome is, therefore, a disease with more rapid tumor progression from a benign, initiated clone to frank malignancy (Figure 34-4).

Although germline mutations in the known mismatch repair genes have been detected in only 2% to 4% of CRC patients, roughly 15% of all CRCs display the MSI-H phenotype.[47] The somatic inactivation of *MLH1* via epigenetic mechanisms, including hypermethylation of its promoter, seems to be responsible for most MSI-H CRC cases outside of Lynch syndrome families.[47]

Many of the mutations that arise in cells with the MSI-H phenotype may be detrimental to cell growth or may exert no positive selection pressure. However, a subset of mutations could potentially activate oncogenes or inactivate tumor suppressor genes. An example of a gene containing a mononucleotide tract in its coding sequence that is frequently mutated in MSI-H CRCs is the TGF-β type II receptor.[47] Some other genes commonly altered in MSI-H CRCs are described later.

Inflammation and Colon Cancer

Chronic inflammatory bowel disease (IBD), especially ulcerative colitis, confers an increased risk of CRC. In the case of UC, the risk of CRC is associated with both the duration and extent of the inflammatory disease. From a pathogenetic standpoint it is notable that UC-associated CRCs often develop without the formation of a polyp as a precursor lesion, although dysplasia is a common factor.[15,48] Reducing the degree of inflammation in IBC seems to reduce the risk for CRC.[49] Some insights into the factors and pathways by which inflammation contributes to colitis-associated CRC (CAC) have begun to emerge[50] and likely include important roles for NF-κB signaling in both immune cells and epithelial cells and local activation of various cytokines and lymphokines, including tumor necrosis factor, interleukin 1 (IL-1), IL-6, IL-17, and IL-23.[50]

The responses of the innate and cellular immune systems to variations in intestinal microbial communities as well as inflammatory mediators all seem to have important and potentially much broader contributing roles in intestinal neoplasia beyond the setting of CAC. For instance, a human colonic commensal—enterotoxigenic *Bacteroides fragilis*—can affect tumor progression in the *Apc(Min)* mouse model of intestinal tumorigenesis in part via inflammation and the generation of IL-17–expressing T helper 17 [T(H)17] cells.[51] Recent studies have further highlighted the role of microbes and microbial products, along with IL-23 and T(H)17 cells in *Apc*-mutation–dependent colon tumor progression in the mouse.[52] Future studies are likely to further clarify the likely complex relationships among the intestinal microbiota, host immune cells, and epithelial cells in the initiation and progression of colon neoplasia in mouse models and in humans.

Recurrent Somatic Alterations in Colorectal Cancer

Somatic alterations have key roles in the initiation and progression of colorectal tumors, in patients with inherited predispositions to CRC as well as in the majority of CRC patients whose tumors are apparently sporadic. A sizeable number of mutational alterations in CRCs were identified over the past 30 years, largely by various combinations of genetic and genomic approaches.[18] Among the major targets for somatic mutations in CRCs identified before whole genome sequence-based approaches included oncogenic (activating) point mutations affecting *KRAS* (about 40% of CRCs), *NRAS* (about 5%), *PIK3CA* (20%), and *BRAF* (5% to 10%), as well as amplifications of the *EGFR* (5% to 10%), *CDK8* (10% to 15%), *CMYC* (5% to 10%), and *ERBB2* (5%) genes.[18] Similarly, among the tumor suppressor genes known to be frequently affected by somatic mutations in CRCs as a result of the "old-school," focused sequencing approaches were the following: *APC* (80%), *TP53* (60%), *FBXW7* (20%), *SMAD4* (10% to 15%), *PTEN* (10%), *SMAD2* (5% to 10%), *ACVR2A* (10%), and *TGFBR2* (10%).[18]

Over the past few years, comprehensive sequencing approaches have allowed for expansive analyses of the nature and spectrum of mutational alterations in CRCs.[17,19,20] One of the major advances of the comprehensive sequencing-based approaches has been knowledge of the variation in total somatic mutations among CRCs. For example, in an exome-sequence analysis of 224 tumor and normal pairs from CRC patients, it was found that 84% of the tumors had a median number of nonsilent (predicted to change protein-coding sequence) somatic mutations of 58 per tumor; the remaining 16% of the tumors were reported to manifest a "hypermutated" phenotype, with a median of 728 somatic nonsilent mutations per tumor.[19] These findings that about 16% of CRCs had a hypermutated phenotype was not entirely unexpected. As described earlier in the discussion of mismatch repair gene defects in Lynch syndrome and also apparently sporadic CRCs, about 15% of all CRCs manifest the MSI-H phenotype, in the majority of MSI-H CRCs because of epigenetic silencing of the *MLH1* gene.[18,47] As a result of their defective mismatch repair pathway function, the MSI-H CRCs were known to have a hypermutable phenotype. However, what was a bit unexpected about the hypermutated CRCs identified via the comprehensive sequencing approaches was that about 25% of the hypermutated tumors lacked the MSI-H phenotype and had intact function of *MLH1*. Instead, the hypermutated non-MSI-H cases had somatic mutations in other mismatch repair genes (e.g., *MSH2*, *MSH3*, *MLH3*, *MSH6*, *PMS2*) or in the *POLE* gene encoding DNA polymerase ε.[19] One somewhat puzzling issue is why somatic mutations in certain mismatch repair genes do not invariably lead to demonstrable instability in the microsatellite sequences that define the MSI-H phenotype, whereas epigenetic silencing of MLH1 does apparently lead to the MSI-H phenotype in almost all cases.

Table 34-2 Localized Somatic Mutations in Oncogenes and Tumor Suppressor Genes in Hypermutated and Non hypermutated CRCs

Gene	Estimated Mutation Frequency*
Hypermutated Tumors	
Oncogenes	
BRAF	40%-45%
Tumor Suppressor Genes	
ACVR2A	60%-65%
APC	50%-55%
TGFBR2	50%-55%
MSH3	40%
MSH6	40%
MYO1B	30%-35%
TCF7L2	30%-35%
Nonhypermutated Tumors	
Oncogenes	
KRAS	40%-45%
PIK3CA	15%-20%
NRAS	5%-10%
CTNBB1	5%-10%
Tumor Suppressor Genes	
APC	80%-85%
TP53	60%
FBXW7	10%-15%
SMAD4	10%
TCF7L2	5%-10%
FAM123B	5%-10%
SMAD2	5%-10%

*Mutation estimates from References 18 and 19.

Perhaps part of the explanation might be the relative timing of the defects in mismatch repair function in the natural history of a given CRC.

Besides clarifying the mutation rate in CRCs and illuminating the molecular basis for the markedly increased mutation rate in the roughly 16% of CRCs that are hypermutated, comprehensive molecular characterization efforts have yielded additional insights, such as refinements in the estimated frequencies for known oncogene and tumor suppressor gene defects in CRC, the identification of some new oncogene and tumor suppressor gene defects contributing to CRC, and some initial insights into how epigenetic changes and gene expression patterns may be associated with certain mutation patterns as well as biologic and clinicopathologic subsets of CRC. Table 34-2 summarizes some of the genes most frequently affected by somatic mutations in nonhypermutated and hypermutated CRCs.[19,20]

In addition to the extensive data on localized mutation frequencies, comprehensive molecular characterization efforts have begun to further inform understanding of recurrent gene amplification, deletion, and translocation in CRCs. Besides largely confirming the gene amplification events previously uncovered in CRCs, such as *CDK8*, *EGFR*, *CMYC*, and *ERBB2* gene amplifications,[18] the work uncovered gene amplification and overexpression of the *IGF2* gene in about 10% to 15% of CRCs.[19,20] Gene deletions affecting well-defined tumor suppressor genes were confirmed in a subset of CRCs, such as those affecting *APC*, *PTEN*, or *SMAD3*.[19,20] In addition, frequent deletions affecting the *FHIT* presumptive tumor suppressor gene were seen in about 20% to 30% of CRCs.[19,20] Recurrent translocations activating two different genes encoding R-spondin protein family members that cooperate with Wnt ligands to activate canonical β-catenin-dependent Wnt signaling were seen in 5% to 10% of CRCs.[20]

Genetic Instability: Chromosomal Instability versus DNA Mismatch Repair Deficiency

In general, the roughly 15% of CRCs that manifest the MSI-H phenotype tend to have largely diploid or near-diploid karyotypes, whereas the remaining 85% of CRCs are aneuploid.[53,54] Key factors and mechanisms underlying the numerical chromosome instability (CIN) phenotype in CRC and other cancers are poorly defined, but some clues have emerged. Rare mutations and more commonly changes in the expression of genes that encode proteins that regulate chromosome segregation in mitosis or other genes that encode DNA repair response proteins have been suggested to play roles in the CIN phenotype in CRC.[54] However, because the mutations appear to be quite rare in primary CRCs and the changes in gene expression observed have been difficult to conclusively implicate in the CIN phenotype, unambiguous data on the gene defects with significant roles in the CIN phenotype in CRC are lacking.[54]

Recently, three genes from a region of chromosome 18q affected by frequent copy number loss in CRC—PIGN, MEX3C, and ZNF516—have been implicated as potential contributing factors in CIN.[55] Specifically, experimental silencing of any one of the three genes led to the CIN phenotype in CRC cells in culture.[55] The genes appear to regulate the cellular response to DNA replication stress, and reduced DNA replication stress appears linked to reduced chromosome segregation defects.[55] Nonetheless, although the recent work on these three chromosome 18q genes appears to be an encouraging lead in terms of defining

common molecular defects that may play important contributing roles in the CIN phenotype in CRC, much further work is needed to substantiate the contribution of the genes as well as the means by which loss of their function might lead to CIN.

Epigenetic Changes in Colorectal Carcinogenesis: the CpG Island Hypermethylation Phenotype

In mammalian genomes, most 5′-CpG-3′ dinucleotides have been lost during evolution.[56] DNA methylation covalently modifies more than 80% of the remaining CpG sites, except for localized regions of high CpG-dinucleotide content, which have been termed *CpG islands*.[56] The promoter and proximal regulatory regions of about 50% of all genes contain CpG islands. Dense methylation or hypermethylation of these CpG islands seems to be associated with gene silencing, implicating CpG methylation as a potentially quite significant epigenetic mechanism to reinforce or "lock in" long-term gene silencing following more transient posttranslational chromatin modifications that are linked to inhibition of transcription (e.g., certain histone tail methylation and deacetylation changes).[56] Although the global trend in the majority of CRCs is in fact a decrease in total DNA methylation—that is, global DNA hypomethylation—the CpG island hypermethylation of selected promoters has been implicated in transcriptional silencing in CRC and other cancers.[56,57] Indeed, it is likely that a large fraction of CRCs reduce or silence expression of one or more critical growth-controlling genes (e.g., tumor suppressor genes) in part through epigenetic mechanisms, such as promoter DNA hypermethylation.[57]

The functional significance of global DNA hypomethylation in CRC is more enigmatic than DNA hypermethylation, but some findings have suggested a potential contributing role for global DNA hypomethylation in chromosome missegregation events and the CIN phenotype.[57] Other work has suggested that global DNA hypomethylation might also lead to altered gene expression, such as the reactivation of certain imprinted genes that are normally silenced during early development.[18]

A subset of CRCs manifest DNA hypermethylation at a sizeable number of distinct CpG islands scattered around the genome, compared to the DNA methylation patterns seen in adjacent normal colon epithelium or the bulk of other CRCs that lack the significant increase in DNA methylation. This phenotype has been termed the *CpG island hypermethylation phenotype* (CIMP).[57,58] A subset of CIMP CRCs shows hypermethylation of the *MLH1* mismatch repair gene, and this group of CRCs constitutes the major fraction of sporadic MSI-H CRCs discussed at several earlier points. The specific factors and mechanisms that underlie CIMP status in CRC are not understood. However, it is noteworthy that the MSI-H CIMP subset of CRCs often harbors *B-RAF* oncogenic mutations.[9,10,57]

Altered Gene Expression in Colorectal Cancer

Besides the genes whose expression is affected by DNA methylation, many other genes show major differences in gene expression in CRCs relative to normal tissues as well as among CRCs. This is likely a result of complex and heterogeneous mechanisms, such as the following: (1) somatic mutations deregulating key cellular signaling pathways that affect downstream gene expression in CRCs, along with significant intertumoral heterogeneity among CRCs with respect to the specific collection of signaling pathways and factors affected by oncogene and tumor suppressor gene mutations in any given CRC; (2) a subset of CRCs with mutations or epigenetic defects affecting specific chromatin regulatory factors, such as ARID1A[19,58]; and (3) potential intertumoral heterogeneity in the specific cell of origin, with some CRCs arising from presumptive crypt base columnar stem cells and other CRCs potentially arising from more differentiated cell types. As a result of the potential differences in cell of origin and/or the specific signaling pathways and chromatin regulatory factors that are dysregulated in one CRC versus another, there are likely to be major variations among CRCs in terms of the expression of key transcriptional activators and repressors that directly regulate gene expression. There are also likely to be major variations in the structure and posttranslational modifications of chromatin and chromatin-associated proteins in CRCs. It will not be possible to review in any detail the very large collection of published studies on differentially expressed genes in CRC relative to normal tissues or the variations in gene expression among different subsets of CRC. Rather, one goal here is to offer some thoughts about the potential significance of gene expression changes in CRC that are not directly linked to specific mutations. A second goal is to introduce the notion that gene expression changes in CRCs affect not only mRNAs encoding proteins, but also various noncoding RNAs, such as miRNAs and long noncoding RNAs (lncRNAs).

The key somatic mutations in CRC highlighted earlier are clonal and recurrent, and the mutations lead to novel or increased function of oncogenes or inactivation of tumor suppressor genes. As described for the *MLH1* gene, epigenetic changes, such as promoter hypermethylation and perhaps posttranslational modifications of histone or other chromatin-associated proteins, silence *MLH1* expression in nearly all of the apparently sporadic MSI-H CRCs. Based on this powerful example, it could be that many other genes with dysregulated expression in CRC—either increased or decreased expression—might have a functionally significant role as an oncogene or TSG, respectively. However, the altered expression of many genes in CRCs may largely reflect

rather than cause the altered growth and differentiation properties of cancer cells compared to normal cells. Hence, all available data on the function of any differentially expressed gene must be evaluated to establish whether the gene likely has a major contributing role in CRC and whether it might appropriately be designated as an oncogene or tumor suppressor gene.

The stability of mRNA transcripts as well as their ability to be translated into proteins is regulated by microRNAs (miRNAs), which are processed from longer precursor transcripts by the Drosha and Dicer proteins to roughly 18 to 24 nucleotides in length. Recognition of target transcripts predominantly occurs by binding of the miRNA to imperfect complementary sequences in the 3′ untranslated region (3′UTR) of various transcripts. Not unexpectedly, because the primary RNA transcripts that are ultimately processed to mature miRNAs are transcribed by RNA polymerase II (as are mRNAs), miRNA levels in CRCs vary considerably from levels in normal colonic mucosa.[57] miRNA expression differences have been reported in comparisons of MSS CRCs to MSI-H CRCs.[57,59] Many of the reported differences in miRNA levels in CRCs versus normal colon tissues and among different subsets of CRCs may be due to differential expression of the primary transcripts that are processed to miRNAs. However, p53 missense mutations have been hypothesized to exert specific effects on the processing of certain miRNAs.[60] Other somatic mutations in CRC could also conceivably exert major effects on the processing and/or nuclear-cytoplasmic transport of miRNAs. Similar to the situation discussed earlier for protein-coding mRNAs, in most cases it is largely uncertain which miRNA changes have causal roles in CRC development and which are secondary events associated with the process. Unlike the situation in some other cancer types where the genomic location of certain miRNAs with altered expression maps close to recurrent chromosomal breakpoints or deletions, few if any of the miRNAs with altered expression in CRC have been linked to recurrent chromosome lesions in CRC.[61] Besides the likely contribution of oncogene and TSG defects to changes in the levels of various miRNAs in CRC, it is likely that the expression of key components in critical oncogene and TSG pathways in CRC is modulated by many different miRNAs. Finally, although there are no conclusive data to implicate alterations in the function or expression of lncRNAs in CRC development and progression, it seems likely that at least a few of the estimated 5000 or more lncRNAs will have major contributing roles in CRC, such as through the ability of some lncRNAs to function as scaffolds for various chromatin-regulatory proteins.[62] Similar to the situation for both protein-encoding mRNAs, critical assessment of the collective expression and functional data will be required to implicate any given miRNA or lncRNA as a causal factor in CRC development and progression.

Multistep Genetic Models of Colorectal Tumor Development

Based on the accumulation of selected somatic oncogene and tumor suppressor gene defects together with methylation changes in CRCs, an initial genetic model of colorectal tumorigenesis was proposed more than 20 years ago.[63] The model relied on the assumption that many carcinomas arise from preexisting adenomas and that certain genetic alterations tended to accumulate at particular stages of tumor development, such as APC mutations in adenoma formation and p53 mutations in the transition to carcinomas. Hence, this was the basis for assigning a relative order to the alterations in a multistep pathway. However, the order of the mutations in the development of any given CRC was not viewed as invariant, as a few small adenomas with *p53* mutations had been identified and *KRAS* mutations were sometimes associated with progression to carcinoma in some late-stage adenomas.[18,63]

Since the initial genetic model for CRC development was proposed, other distinct molecular pathways to CRC have been suggested, some of which involve other precursor lesions than adenomatous polyps.[9,10] Some of the alternative molecular scenarios are presented in Figure 34-5. The initial genetic model suggested in Reference 63 is outlined at the far right as one of the so-called conventional pathways. As indicated in Figure 34-5, the roughly 2% to 4% of CRC cases arising in individuals carrying germline mutations in a DNA mismatch repair (MMR) gene—Lynch syndrome—likely share some similarities with conventional pathway lesions in terms of the somatically acquired gene lesions that give rise to adenomatous lesions, such as *APC* mutations. As for the approximately 10% to 12% of apparently sporadic cancers that manifest the MSI-H phenotype, many of these are presumed to arise from serrated adenomatous lesions, such as sessile serrated adenomas (SSAs).[9,10] Some of the molecular lesions associated with the genesis of SSAs and the subsequent progression of SSAs to CRCs are distinct from those somatic defects that contribute to the conventional CRCs and the Lynch-type MSI-H CRCs, including frequent *B-RAF* mutations and the silencing of certain genes via promoter hypermethylation (i.e., CIMP CRCs). Finally, though not depicted in Figure 34-5, it is worth noting that the nature and order of mutational events seem to be different in UC-associated CRCs than in sporadic CRCs. For instance, *p53* mutations are typically observed at an earlier time point, perhaps even occurring in the nonneoplastic inflamed mucosa of some patients.[48]

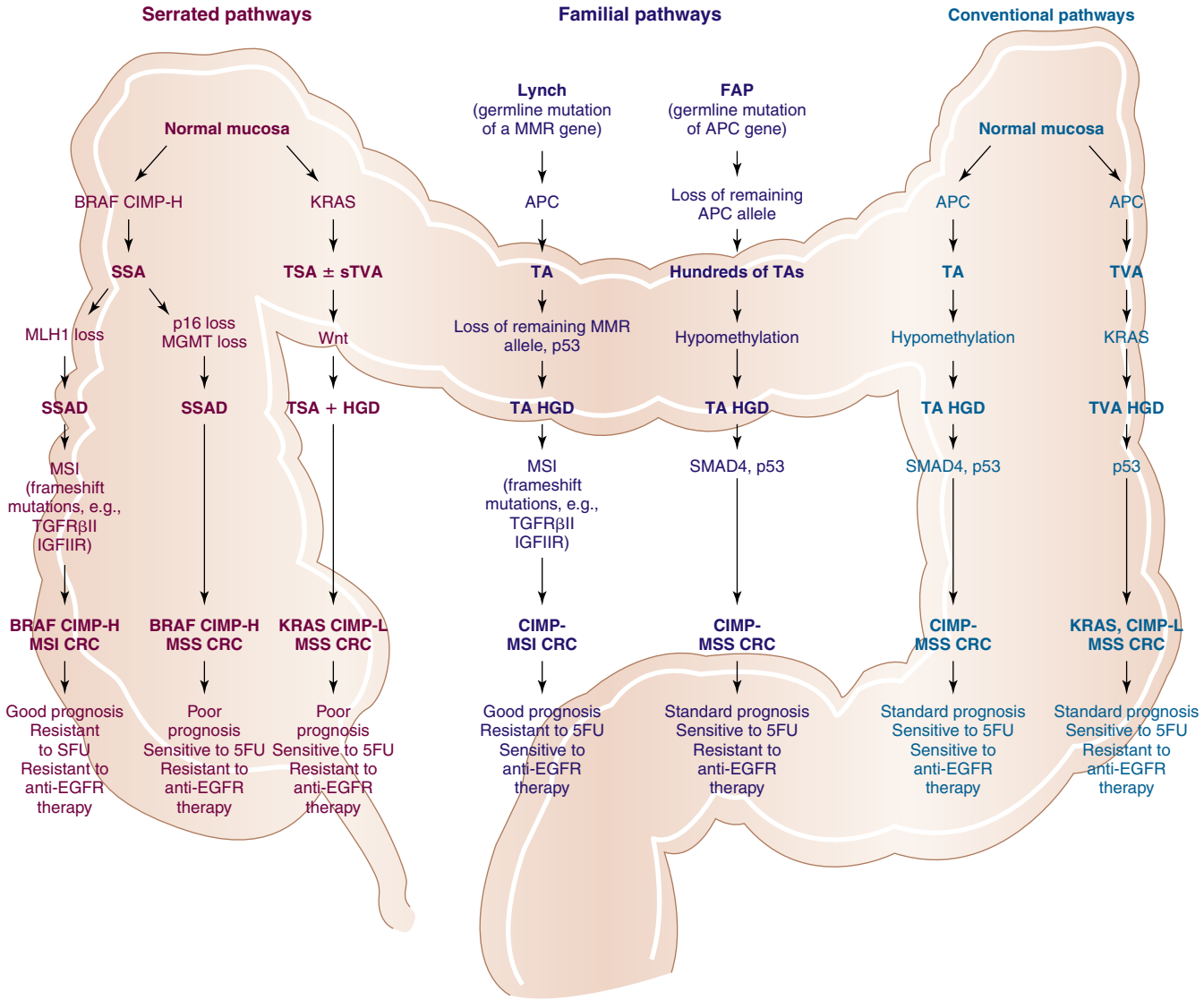


Figure 34-5 Putative pathways to colorectal cancer (CRC) Shown in the figure are potential precursor lesions and selected molecular defects suggested to play important roles in the serrated pathways, familial pathways, or conventional pathways for CRC. The conventional pathways highlight the fact that the majority of CRCs are believed to arise from adenomatous precursor lesions over a period of years or even decades, with progression from a TA or TVA lesion to one that has high grade-dysplasia (TA HGD or TVA HGD). Some of the potential somatic alterations in tumor suppressor genes (APC, SMAD4, p53) and oncogenes (KRAS) that might contribute to tumor initiation and/or progression are illustrated. Possible global phenotypic changes in the CRC cells that are discussed in the text are noted, such as CpG island methylator phenotype-negative (CIMP-) or CIMP-low (CIMP-L) and microsatellite stable (MSS), along with the potential prognostic and therapeutic generalizations. Two familial pathways to CRC that together account for about 2% to 4% of CRC are highlighted in the scheme. The role of gene lesions and presumptive mutational mechanisms are extensively discussed in the text. The figure indicates the relationship of molecular lesions to prognosis and therapy response. A significant fraction of CRCs, especially lesions in the proximal colon, are thought to arise from a serrated precursor lesion, such as a sessile serrated adenoma (SSA) or a traditional serrated adenoma (TSA), with progression through lesions that manifest dysplasia or high-grade dysplasia (SSAD and TSA + HGD). A sizeable subset of SSAs and the resultant CRCs that arose from SSAs manifest the CIMP-high (CIMP-H) phenotype. The possible relationships of the various serrated pathway lesions to prognosis and therapeutic response are noted. *anti-EGFR,* Epidermal growth factor receptor inhibitors; *5-FU,* 5-fluorouracil therapy; *IGFIIR,* insulin-like growth factor receptor II; *p16,* p16 inhibitor of cyclin-dependent kinase 4; *TA,* tubular adenoma; *TGFRβII,* TGF-β receptor II; *TVA,* tubulovillous adenoma. *With permission from Bettington et al. The serrated pathway to colorectal cancer: current concepts and challenges. Histopathology. 2013; 62:367-386.*

Clinical Applications of Molecular Genetic Insights

The advances in our understanding of inherited and somatic defects in CRCs have made possible certain clinical applications and have highlighted the potential for additional clinical strategies that collectively should greatly improve the diagnosis and care of patients and families affected by CRC. Although many possible future clinical applications can be envisioned, just a few that are being actively pursued are described in the following sections.

Risk Assessment

The accurate presymptomatic diagnosis of FAP or Lynch syndrome is of significant value to individuals and families affected by these syndromes. The identification of germline mutations in the APC gene in more than 80% of families with FAP and Gardner syndrome provides the basis for genetic counseling and clinical management of families and individuals at risk for polyposis.[4,5] Similar major progress has been made in defining the germline mutations in Lynch syndrome patients and families.[4,5] Given the progress reviewed here and summarized in Table 34-1 in defining other genetic variants that confer a significantly increased risk of CRC development, the early identification of individuals and families at greatly elevated risk of CRC should lead to improved clinical management of such individuals and families, in large part via the incorporation of optimal screening and prophylactic surgery approaches and potentially even new chemopreventive agents and regimens.

Early Detection

The results of clinical trials indicate that the colonoscopic removal of larger (greater than 1 cm) adenomas and early CRCs has a major impact on CRC incidence.[8] The development of highly specific and sensitive molecular tests for early detection of CRC is an important goal, given the reduced specificity and sensitivity of current noninvasive tests, such as fecal occult blood testing.[8] If inexpensive and reliable molecular diagnostic tests could be developed to detect the presence of advanced adenomas or early-stage CRCs, such as through the analysis of analytes in the blood or in stool specimens, then such tests might serve an adjunctive role along with more invasive and expensive methods for early detection, such as colonoscopy. Findings from studies of DNA isolated from stool samples of patients known to have CRCs or large, advanced adenomas indicate that stool-based tests for mutant oncogenes and tumor suppressor genes may have utility.[64] More recent stool-based DNA tests incorporate detection of mutant KRAS alleles and DNA methylation of specific sequences, along with hemoglobin quantification, and the results seem potentially quite promising compared to earlier generation stool DNA tests.[65] Some early work analyzing DNA methylation of multiple genes in cell-free DNA in plasma from patients with colorectal cancer and adenomatous polyps suggests the future potential for using a panel of DNA methylation and/or somatic mutation markers for screening.[66] Extensive further studies are needed to clarify whether any plasma-based tests for circulating nucleic acid markers (e.g., DNA hypermethylation and somatic mutations) are strong competitors for the stool-based DNA tests and what the optimal strategies will be for deploying any stool-based and/or plasma-based DNA tests in populations at high risk and/or average risk for adenomas and CRC.

Prognostic and Predictive Markers

In addition to presymptomatic diagnosis (risk assessment) and early detection of tumors, several studies indicate that characterization of the specific genetic alterations in a cancer may provide improved/increased prognostic information about the likelihood of local and distant tumor recurrence. Perhaps among the most robust prognostic markers defined thus far for colorectal cancer are those for the MSI-H phenotype. In particular, this phenotype has been convincingly associated with improved survival in stage II and stage III colorectal cancer patients.[67] Interestingly, the use of 5-fluorouracil (5-FU)-based adjuvant chemotherapy did not appear to show any benefit in survival for patients with MSI-H tumors.[68] In fact, although not a statistically significant result in the initial study, the trend was for poorer survival in 5-FU–treated patients whose tumors displayed the MSI-H phenotype. Some of the data on molecular pathways to CRC and the relationship of the pathways and somatic alterations to prognosis and response to therapies are highlighted in Figure 34-5. Moreover, a number of studies have suggested that oncogenic mutations in KRAS are associated with resistance to EGFR-based therapies,[69] and some recent studies indicate that there is strong biologic selection for the outgrowth of KRAS-mutant CRCs when EGFR blockade is used therapeutically in CRC.[70]

Summary and Future Directions

Molecular genetic studies of colorectal tumors have yielded profound insights into inherited predispositions to colorectal cancer as well its pathogenesis. A relatively limited number of oncogenes and tumor suppressor genes—the KRAS, APC, and TP53 genes, and a few others—have been found to be frequently mutated in CRCs, and intensive studies of the function of these critical genes in normal and neoplastic cell growth continue. The relative significance to the cancer cell phenotype of each of the various inherited and somatic mutations has not been well defined. Comprehensive sequence analyses indicate that a considerable number of additional oncogenes and tumor suppressor genes with roles in the development and progression of subsets of CRC likely exist. Identification of these genes and characterization of their contribution to cancer will be an important, albeit a challenging, task. At present, there is little understanding of

the relationship between dietary and environmental agents associated with increased risk of colorectal cancer and the mutation rate and nature of the mutations that arise in normal and neoplastic cells in the colon and rectum. Only limited insights have been obtained into the true significance and generality of the findings from the clinical correlative studies undertaken to date. Nevertheless, it is clear that further efforts will yield insights into the molecular basis of CRC and can be expected to result in advances in the diagnosis and clinical care of patients with colorectal tumors.

Key References

10. Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology*. 2013;62:367-386.
18. Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol Mech Dis*. 2011;6:479-507.
19. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487:330-337.
20. Seshagiri S, Stawiski EW, Durinck S. Recurrent R-spondin fusions in colon cancer. *Nature*. 2012;488:660-664.
31. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
54. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology*. 2010;138:2059-2072.
57. Goel A, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology*. 2012;143:1442-1460.
70. Diaz Jr LA, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012;486:537-540.

This page intentionally left blank

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA: Cancer J Clin*. 2013;63:11-30.
2. Polite BN, Dignam JJ, Olopade OI. Colorectal cancer model of health disparities: understanding mortality differences in minority populations. *J Clin Oncol*. 2006;24:2179-2187.
3. Dimou A, Syrigos KN, Saif MW. Disparities in colorectal cancer in African-Americans vs whites: before and after diagnosis. *World J Gastroenterol*. 2009;15:3734-3743.
4. Jasperson KW, Tuohy TM, Neklason DW, et al. Hereditary and familial colon cancer. *Gastroenterology*. 2010;138:2044-2058.
5. Patel SG, Ahnen DJ. Familial colon cancer syndromes: an update of a rapidly evolving field. *Curr Gastroenterol Rep*. 2012;14:428-438.
6. Chan AT, Giovannucci EL. Primary prevention of colorectal cancer. *Gastroenterology*. 2010;138:2029-2043.
7. Watson AJM, Collins PD. Colon cancer: a civilization disorder. *Dig Dis*. 2011;29:222-228.
8. Ransohoff DF. Colon cancer screening in 2005: status and challenges. *Gastroenterology*. 2005;128:1685-1695.
9. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology*. 2007;50:113-130.
10. Bettington M, Walker N, Clouston A, et al. The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology*. 2013;62:367-386.
11. Murakami R, Tsukuma H, Kanamori S, et al. Natural history of colorectal polyps and the effect of polypectomy on occurrence of subsequent cancer. *Int J Cancer*. 1990;46:159-164.
12. Winawer SJ, Zauber AG, Fletcher RH, et al. Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society. *Gastroenterology*. 2006;130:1885.
13. Lin OS, Gerson LB, Soon MS, et al. Risk of proximal colon neoplasia with distal hyperplastic polyps: a meta-analysis. *Arch Int Med*. 2005;165:382-390.
14. Lin OS, Schembre DB, McCormick SE, et al. Risk of proximal colorectal neoplasia among asymptomatic patients with distal hyperplastic polyps. *Am J Med*. 2005;118:1113-1119.
15. Ullman T, Croog V, Harpaz N, et al. Progression of flat low-grade dysplasia to advanced neoplasia in patients with ulcerative colitis. *Gastroenterology*. 2003;125:1311-1319.
16. Soetikno R, Friedlnad S, Kaltenbach T, et al. Nonpolypoid (flat and depressed) colorectal neoplasms. *Gastroenterology*. 2006;130:566-576.
17. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
18. Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol Mech Dis*. 2011;6:479-507.
19. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487:330-337.
20. Seshagiri S, Stawiski EW, Durinck S. Recurrent R-spondin fusions in colon cancer. *Nature*. 2012;488:660-664.
21. Polakis P. Mutations in the APC gene and their implications for protein structure and function. *Curr Opin Genet Dev*. 1995;5:66-71.
22. Hamilton SR, Liu B, Parsons RE, et al. The molecular basis of Turcot's syndrome. *N Engl J Med*. 1995;332:839-847.
23. Davies DR, Armstrong JG, Thakker N, et al. Severe Gardner syndrome in families with mutations restricted to a specific region of the APC gene. *Am J Hum Genet*. 1995;57:1151-1158.
24. Niewuwenhuis MH, Vasen HF. Correlation between mutation site in APC and phenotype of familial adenomatous polyposis (FAP): a review of the literature. *Crit Rev Oncol Hematol*. 2007;61:153-161.
25. Brosens LA, Keller JJ, et al. Prevention and management of duodenal polyps in familial adenomatous polyposis. *Gut*. 2005;54:1034-1043.
26. Gallagher MC, Phillips RK, Bulow S. Surveillance and management of upper gastrointestinal disease in familial adenomatous polyposis. *Fam Cancer*. 2006;5:263-273.
27. Laken SJ, Petersen GM, Gruber SB, et al. Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC. *Nat Genet*. 1997;17:79-83.
28. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell*. 1996;87:159-170.
29. Jen J, Powell SM, Papadopoulos N, et al. Molecular determinants of dysplasia in colorectal lesions. *Cancer Res*. 1994;54:5523-5526.
30. Nathke IS. The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. *Annu Rev Cell Dev Biol*. 2004;20:337-366.
31. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. *Cell*. 2012;149:1192-1205.
32. Morin PJ, Sparks AB, Korinek V, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science*. 1997;275:1787-1790.
33. Wu ZQ, Brabletz T, Fearon E, et al. Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. *Proc Natl Acad Sci U S A*. 2012;109:11312-11317.
34. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science*. 1990;247:322-324.
35. Cormier RT, Hong KH, Halbert RB, et al. Secretory phospholipase Pla2g2a confers resistance to intestinal tumorigenesis. *Nat Genet*. 1997;17:88-91.
36. Laird PW, Jackson-Grusby L, Fazeli A, et al. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*. 1995;81:197-205.
37. Lin H, Yamada Y, Nguyen S, et al. Suppression of intestinal neoplasia by deletion of Dnmt3b. *Mol Cell Biol*. 2006;26:2976-2983.
38. Oshima M, Dinchuk JE, Kargman SL, et al. Suppression of intestinal polyposis in Apcdelta716 knockout mice by inhibition of cyclo-oxygenase 2 (COX-2). *Cell*. 1996;87:803-809.
39. Peek Jr RM. Prevention of colorectal cancer through the use of COX-2 selective inhibitors. *Cancer Chemother Pharmacol*. 2004;54:S50-S56.
40. Palles C, Cazier JB, Howarth KM, et al. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. *Nat Genet*. 2013;45:136-144.
41. Jaeger E, Leedham S, Lewis A, et al. Hereditary mixed polyposis syndrome is caused by a 40-kb upstream duplication that leads to increased and ectopic expression of the BMP antagonist GREM1. *Nat Genet*. 2012;44:699-703.
42. Warthin AS. Heredity with respect to carcinoma. *Arch Int Med*. 1913;12:546.
43. Lynch HT, Shaw MW, Magnuson CW, et al. Hereditary factors in cancer: study of two large Midwestern kindreds. *Arch Int Med*. 1966;117:206-212.
44. Watson P, Riley B. The tumor spectrum in the Lynch syndrome. *Fam Cancer*. 2005;4:245-248.
45. Peltomaki P, Aaltonen LA, Sistonen P, et al. Genetic mapping of a locus predisposing to human colorectal cancer. *Science*. 1993;260:810-812.
46. Lindblom A, Tannergard P, Werelius B, et al. Genetic mapping of a second locus predisposing to hereditary non-polyposis colon cancer. *Nat Genet*. 1993;5:279-282.
47. Pino MS, Chung DC. Microsatellite instability in the management of colorectal cancer. *Expert Rev Gastroenterol Hepatol*. 2011;5:385-399.
48. Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology*. 2011;140:1807-1816.
49. Beaugerie L. Inflammatory bowel disease therapies and cancer risk: where are we and where are we going. *Gut*. 2012;61:476-483.
50. Grivennikov SI. Inflammation and colorectal cancer: colitis-associated neoplasia. *Semin Immunopathol*. 2013;35:229-244.
51. Wu S, Rhee KJ, Albesiano E, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med*. 2009;15:1016-1022.
52. Grivennikov SI, Wang K, Mucida D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature*. 2012;491:254-258.
53. Lengauer C, Kinzler KW, Vogelstein G. Genetic instability in human cancers. *Nature*. 1998;396:643-649.
54. Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology*. 2010;138:2059-2072.
55. Burrell RA, McClelland SE, Endesfelder D, et al. Replication stress links structural and numerical cancer chromosome instability. *Nature*. 2013;494:492-496.
56. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012;13:484-492.
57. Goel A, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology*. 2012;143:1442-1460.
58. Jones S, Li M, Parsons DW, et al. Somatic mutations in the chromatin remodeling gene ARID1A occur in several tumor types. *Hum Mutat*. 2012;33:100-103.

59. Lanza G, Ferracin M, Gafa R, et al. mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol Cancer*. 2007;6:54.
60. Suzuki HI, Yamagata K, Sugimoto K, et al. Modulation of microRNA processing by p53. *Nature*. 2009;460:529-533.
61. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435:834-838.
62. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem*. 2012;81:145-166.
63. Fearon ER. Vogelstein. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61:759-767.
64. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med*. 2004;351:2704-2714.
65. Ahlquist DA, Zhou H, Domanico M, et al. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology*. 2012;142:248-256.
66. Cassinotti E, Melson J, Liggett T, et al. DNA methylation patterns in blood of patients with colorectal cancer and adenomatous polyps. *Int J Cancer*. 2012;131:1153-1157.
67. Gryfe R, Kim H, Hsieh ET, et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med*. 2000;342:69-77.
68. Carethers JM, Smith EJ, Behling CA, et al. Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. *Gastroenterology*. 2004;126:394-401.
69. Markman B, Javier Ramos F, Capdevila J, et al. EGFR and KRAS in colorectal cancer. *Adv Clin Chem*. 2010;51:71-119.
70. Diaz Jr LA, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012;486:537-540.
71. Bettingon et al. The serrated pathway to colorectal cancer: current concepts and challenges. *Histopathology*. 2013;62:367-386.

Rachna T. Shroff and James L. Abbruzzese

35

Molecular Pathogenesis of Pancreatic Adenocarcinoma

The most common exocrine pancreatic neoplasm is pancreatic ductal adenocarcinoma, which accounts for more than 95% of all pancreatic malignancies. Other pancreatic malignant neoplasms include acinar cell carcinoma, serous cystadenocarcinoma, mucinous cystadenocarcinoma, intraductal papillary-mucinous neoplasm, osteoclast-like giant cell tumor, solid pseudopapillary carcinoma, and pancreatoblastoma.

It has been hypothesized that the development of pancreatic adenocarcinoma follows progressive stages of neoplastic growth through precursor lesions to adenocarcinoma, similar to the models proposed for colorectal cancer and prostate cancer. The precursor lesions are best defined for invasive ductal adenocarcinoma and have been termed *pancreatic intraepithelial neoplasia* (PanIN). PanINs are characterized by architectural changes manifested by change of the normal cuboidal epithelium to a columnar epithelium, nuclear hyperchromasia and atypia, loss of epithelial polarity, pseudostratification and papillary folding, and eventually to carcinoma in situ and invasive carcinoma (Figure 35-1[1]).

Molecular Alterations in Pancreatic Cancer

Pancreatic carcinogenesis is driven by multiple genetic and epigenetic events, including inactivation of tumor-suppressor genes and activation of proto-oncogenes. Table 35-1 lists the most frequently identified genetic alterations. K-*ras* mutation is believed to be an early genetic event, followed by loss of functional *p16*, p53, SMAD4, and many other changes.

Whole-exome sequencing of 24 different pancreatic cancer specimens at Johns Hopkins University has helped further elucidate some of the "driver" genes in pancreatic carcinogenesis.[2] More than 20,000 genes were sequenced from these tumors, representing 99.6% of the coding genome of pancreatic cancer, and from this 69 sets of genes were identified as being altered in the majority of the 24 specimens. Of these tumors, 67% to 100% had genetic alterations that could be clustered into 12 signaling pathways and processes felt to be pivotal in tumorigenesis (Figure 35-2). The specific genes altered in these pathways varied greatly among samples; however, only one gene in each of these pathways was altered in each tumor. This suggests that there are core pathways that play a role in pancreatic cancer, and that understanding these alterations will provide further insight into pancreatic cancer biology.

Work from the rapid autopsy program in pancreatic cancer that was established by Iacobuzio-Donahue and colleagues at Johns Hopkins University has further identified various genetic subtypes of pancreatic cancer based on clinical and pathologic features.[3] Seventy-six patients underwent rapid autopsy, with 88% having metastatic disease at the time of death. Interestingly, the metastatic burden varied greatly among these patients. Genetic analysis of K-*ras*, p53, and *SMAD4* status demonstrated that patients with a higher volume of metastatic disease more commonly had a loss of *SMAD4* expression. These findings need to be confirmed with additional studies, but they imply that specific molecular features of pancreatic primary tumors influence the behavior of this disease as it progresses over time. This chapter focuses on some of the key molecular changes that characterize exocrine pancreatic cancer.[4]

Events Targeting Signal Transduction Pathways

K-*ras* Mutation

K-*ras* mutations can be detected in approximately 30% of early PanINs and increase in frequency with disease progression. K-*ras* mutations can be identified in nearly 95% of invasive ductal pancreatic adenocarcinomas.[5] The early onset of K-*ras* mutations supports a role in tumor initiation.

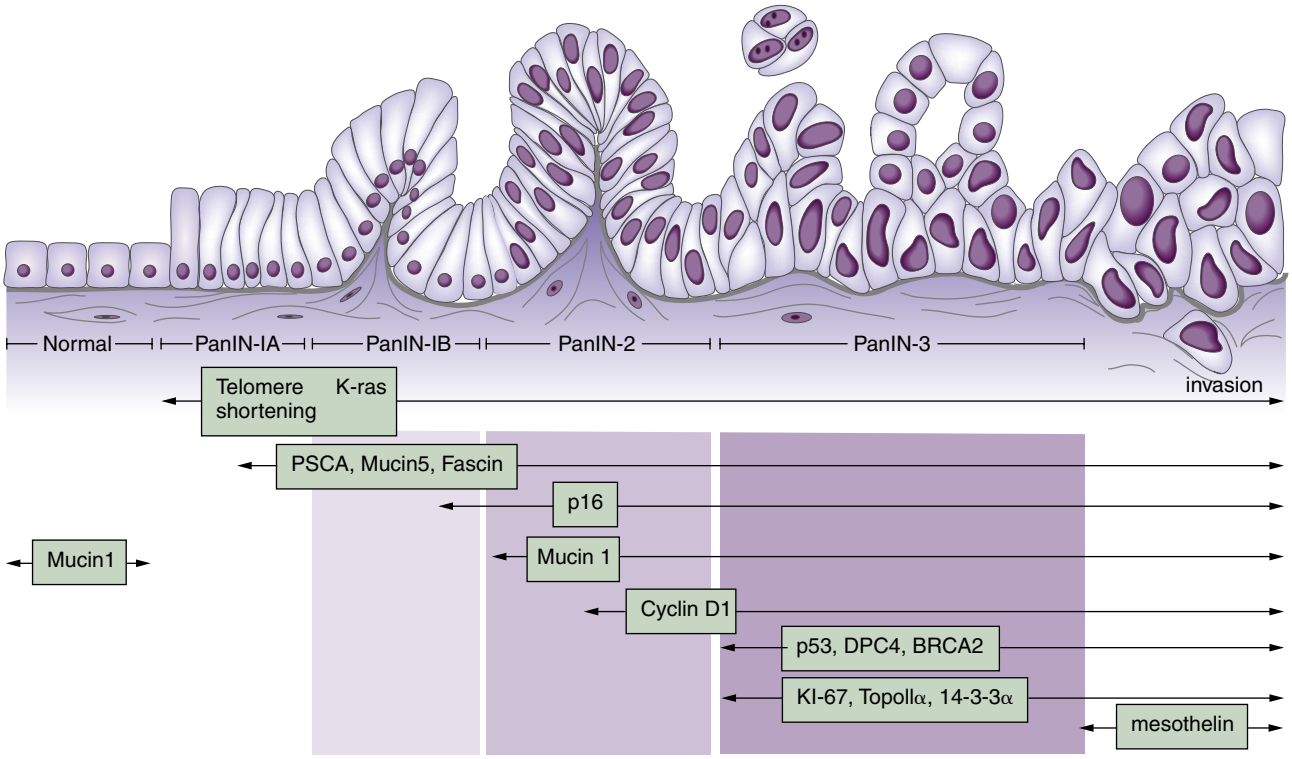


Figure 35-1 Pancreatic epithelial neoplasia and the multistep model of exocrine pancreatic cancer. *(From Maitra A, Adsay NV, Argani P, et al. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray.* Mod Pathol. *2003;16:902-912, with permission.)*

Table 35-1 Frequent Molecular Alterations in Pancreatic Adenocarcinoma

Molecular Alteration	Frequency of Event in Exocrine Pancreatic Cancer, %
Oncogene Activation	
K-ras	90
RTK Overexpression	
EGFR	95
HER2	10
Tumor Suppressors	
p^{INK4A}	95
P53	55
$SMAD_4$	50
PTEN	60
Transcription Factor Activation	
NF-κB	67

RTK, Receptor tyrosine kinase.

Transgenic mouse models have provided further evidence that support this notion. A first-generation transgenic mouse model was generated in which K-*ras* was driven by an elastase promoter and the resultant transgene was expressed in pancreatic acinar cells. Transgenic mice bearing this transgene exhibit acinar hyperplasia, acinar-ductal metaplasia, and noninvasive intrapapillary mucinous neoplasia (IPMN). Frequently, these lesions were accompanied by focal dysplasia, fibrosis and/or lymphocytic infiltration, and occasional carcinoma in situ.[6] PDX-1 and p48 are critical transcription factors in early pancreas development, and these genes have been employed in most recent transgenic mouse models. Hingorani and colleagues generated a mouse model in which K-*ras*(G12D) mice are crossed with mice expressing Cre-recombinase through PDX-1 or p48 promoters. The result is a heterozygous mutant mouse, K-*ras*(+/G12D) that recapitulates the temporal sequential development of high-grade PanIN lesions with a small percentage progressing to invasive and metastatic adenocarcinoma.[7] Most lesions in this model appear arrested at a preinvasive stage despite confirmed expression of the K-*ras* gene, suggesting that K-*ras* mutation is not always sufficient to induce progression to invasive pancreatic carcinoma and that other genetic events are required.

To overcome these effects, a second mutation, such as inactivation of tumor suppressor genes, *p16*, *SMAD4*, and/or *p53* may be necessary for the development of invasive/metastatic pancreatic adenocarcinoma. Consistent with this hypothesis, more recent transgenic models have used genetically engineered mice with promoters that are developmentally expressed in progenitors of all pancreatic cell types. For example, genetically engineered mice expressing the mutant K-*ras*(G12D) allele mutation develop focal premalignant

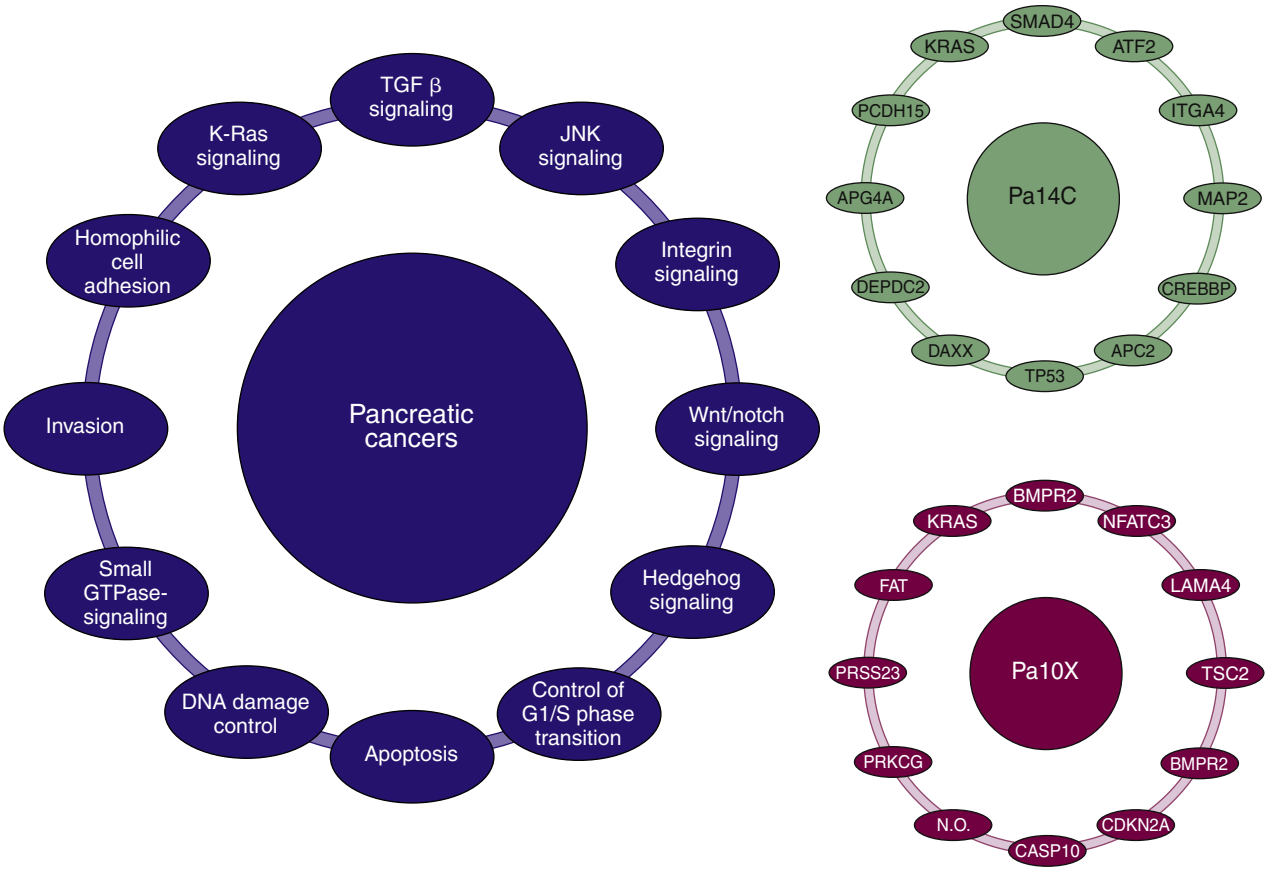


Figure 35-2 Major pathways and processes that are genetically altered in most pancreatic cancers. *From Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science. *2008;321:1801-1806.*

lesions consistent with human PanIN,[8] but a mouse with activation of a mutant K-*ras* allele (*Kras(G12D)*) and deletion of a conditional *Ink4a/Arf* tumor suppressor allele resulted in an earlier appearance of PanIN lesions, and these neoplasms progressed rapidly to highly invasive and metastatic cancers.[9] Similarly, the KPC mouse model was created by interbreeding mice with mutant K-*ras*(G12D) and Trp53(R172H) alleles with the PDX-1-Cre transgenic mice. These mice developed pancreatic adenocarcinoma rapidly and demonstrated a widely metastatic phenotype with a markedly shorter life span.[10] The evolution of these tumors bears striking resemblance to the human disease, possessing a proliferative stromal component and ductal lesions with a propensity to advance quickly. These findings in the mouse provide experimental support for the widely accepted model of human pancreatic adenocarcinoma in which activated K-*ras* serves to initiate PanIN lesions, and other tumor suppressors function to constrain the malignant conversion of these PanIN lesions into lethal ductal adenocarcinoma.

Recently, an inducible, genetically engineered mouse model of oncogenic K-*ras* revealed the integral role that K-*ras* plays in tumor maintenance and metabolism.[11] This mouse model supports the view that advanced pancreatic cancer remains dependent on K-*ras* expression. Furthermore, transcriptomic and metabolomic analyses demonstrated that mutant K-*ras* controls glycolysis by regulating glucose transporters as well as shunting glucose metabolism to anabolic pathways. These findings demonstrate how K-*ras* modifies metabolic pathways in pancreatic tumors to support the high energy requirements of tumor metabolism. These metabolic targets may also serve as novel targets for pancreatic cancer therapy.

Although K-*ras* mutation appears critical to the initiation of human pancreatic carcinogenesis and the initiation and maintenance of pancreatic cancer in genetically engineered mouse models, its role in the maintenance of established human pancreatic adenocarcinoma remains less clear. In addition, although K-*ras* mutation is widely detected in pancreatic adenocarcinoma, its expression can also be detected in nonmalignant conditions such as chronic pancreatitis. Disappointingly, novel therapies that target mutant K-*ras* have not been effective.

Epidermal Growth Factor Receptor

The role for epidermal growth factor receptor (EGFR) and its downstream signaling molecules in tumorigenesis is evidenced by their ability to transform normal cells to a neoplastic phenotype when expressed in mutated, unregulated

forms or when expressed to an abnormally high level. Overexpression of EGFR and its downstream signaling molecules occurs frequently in a variety of human cancers, including pancreatic cancer. A prospective study indicated that EGFR was detectable in more than 95% of patients with advanced pancreatic cancer.[12] In most cases, EGFR is concomitantly expressed with its ligands, EGF or TGF-β. It has been hypothesized that the increased expression of ligand and receptor forms an autocrine loop that constantly stimulates cell proliferation. A study found that pancreatic cancer cell lines display heterogeneous sensitivity to the EGFR inhibitor gefitinib. Three of nine cell lines studied displayed significant sensitivity to pharmacologically relevant concentrations of gefitinib (1 μmol/L) as measured by two independent assays for G_1-S cell cycle arrest.[13] Clinically, erlotinib, an oral small-molecular inhibitor of EGFR, was approved for the treatment of advanced pancreatic cancer based on a study by Moore and colleagues.[14] In this study, 569 patients were treated with either gemcitabine alone or gemcitabine with daily erlotinib. Median overall survival was significantly improved with the addition of erlotinib (6.24 months vs. 5.91 months, $P = .038$). Although the incremental survival benefit is small, it does suggest a role for the EGFR pathway in a subset of patients with pancreatic cancer.

Activation of Nuclear Transcription Factors

Nuclear Factor κB

Nuclear factor κB (NF-κB) is a family of pleiotropic transcription factors that regulate the expression of a spectrum of genes important in growth, oncogenesis, differentiation, and apoptosis. NF-κB proteins are normally sequestered in the cytoplasm in an inactive form through their association with the inhibitor IκBα, which masks the nuclear localization signal (NLS) of NF-κB, thereby preventing its nuclear translocation. NF-κB is activated through activation of the IκB kinase complex (IKK), which phosphorylates IκBα and, as a result of proteasomal degradation, releases NF-κB from the complex, exposing the NLS. Constitutive NF-κB activation has been detected in approximately 70% of pancreatic adenocarcinomas and 9 of 11 human pancreatic tumor cell lines, but not in normal pancreatic tissue.[15]

Many upstream events can potentially affect NF-κB and other transcription factors. PTEN loss in conjunction with oncogenic K-*ras* activates NF-κB and its vast cytokine network. This results in a stromal response and inflammatory infiltration with tumorigenic potential.[16] Furthermore, proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1 induce rapid degradation of IκBα, resulting in nuclear translocation of NF-κB and sustained activation of NF-κB mediated through IκBβ. The EGFR-mediated signaling cascade and Ras signaling pathways may induce constitutive NF-κB activity as well.

Once activated, NF-κB mediates transcription of numerous genes encoding growth factors, cytokines, and apoptotic and cell cycle regulators. The expression of the apoptosis inhibitors that are regulated by NF-κB include c-IAP1, C-IAP2, Traf1, Traf2, A20, IEX-1_L, and the Bcl-2 homologues Bfl-1/A1 and Bcl-x. NF-κB also activates the expression of genes that are important in invasion and metastasis, including matrix metalloproteinases, urokinase plasminogen activator, and ICAM-1. Like NF-κB, numerous other nuclear transcription factors such as AP1,[17] Sp proteins,[18,19] and Stat3[20] have been shown to be activated in exocrine pancreatic cancer. In some cases these proteins are being actively targeted for therapy.[21]

Loss of Tumor Suppressors

INK_4A and *ARF* Tumor Suppressors

Studies have demonstrated that the 9q21 locus encodes two important and overlapping tumor suppressor proteins: *p16*(INK4A) and p19(ARF). Loss of *p16*(INK4A) appears to be critical for the development of exocrine pancreatic cancer, occurring in up to 95% of cases.[22] Genetically, loss of the INK4A locus can occur through mutation, deletion, or promoter hypermethylation. Functionally, loss of *p16*(INK4A) allows CDK4/6 to phosphorylate RB, thereby facilitating entry into the S-phase of the cell cycle. The importance of *p16*(INK4A) in pancreatic carcinogenesis has been highlighted by studies of Bardeesy and co-workers, where *p16*(INK4A) mutations cooperate with mutant K-*ras* and *p53* mutations in the development and progression of exocrine pancreatic carcinomas.[23]

p53

The *p53* tumor suppressor is mutated in at least 50% of patients with exocrine pancreatic cancer and appears to be most important during the later stages of tumor progression.[24] Loss of *p53* may also contribute to the drug resistance and chromosomal instability that are characteristic of pancreatic cancer.

SMAD4

SMAD4 is a tumor suppressor gene.[25] Smad proteins belong to a family of proteins that are part of the TGF-β signaling pathway and negatively regulate the growth of epithelial cells. On binding of TGF-β, TGF-β receptor II activates TGF-β receptor I by phosphorylation. TGF-β receptor I in turn activates Smad2 and Smad3. The activated Smad2 and Smad3 form a hetero-oligomer with Smad4. This Smad complex translocates to the nucleus, where it interacts with

DNA directly or indirectly through other DNA-binding proteins, regulating transcription of target genes. *SMAD4*, also known as DPC4 (homozygously deleted in pancreatic carcinoma locus 4), is frequently deleted or mutated in pancreatic carcinoma. Nearly 90% of pancreatic carcinoma cases show loss of heterozygosity for SMAD4, and 30% to 37% have a homozygous deletion of the *SMAD4* region. In addition, there are intragenic inactivating mutations, including nonsense, missense, and frameshift mutations. In total, approximately 55% of pancreatic carcinomas have deletion or an inactivating mutation of *SMAD4*. Loss of *SMAD4* occurs with a frequency of 10% or less in other malignancies, which suggests a specific role for *SMAD4* in pancreatic carcinogenesis.[26] Inactivation of the *SMAD4* gene correlates with loss of expression of its protein and can be monitored during progression of PanINs. In one study, of 188 PanIN lesions examined, Smad4 was not expressed in 31% of the high-grade lesions, whereas all low-grade PanIN lesions had detectable Smad4 protein.[27] This observation is consistent with the notion that K-*ras* is the initiation factor in pancreatic carcinogenesis followed by alterations of a variety of genes, including *SMAD4*, *p16*, *p53*, and others.

SMAD4 may also be a prognostic factor. Using immunohistochemistry, the SMAD4 protein status of 249 pancreatic adenocarcinomas from patients who underwent pancreaticoduodenectomy was examined. The *SMAD4* gene status of 56 (22%) of 249 pancreatic carcinomas was also determined. It was found that patients with pancreatic adenocarcinomas with Smad4 protein expression had significantly longer survival (unadjusted median survival was 19.2 months as compared with 14.7 months in patients with pancreatic cancers lacking Smad4 protein expression; $p = 0.03$). This Smad4 survival benefit persisted after adjustment for other known prognostic factors including tumor size, margins, lymph node status, pathologic stage, blood loss, and use of adjuvant chemoradiotherapy.[28] These findings may be explained by the correlation between *SMAD4* status and metastatic burden described earlier in this chapter.

PTEN

The tumor suppressor gene *PTEN* is known to play a major role in embryonic development, cell migration, and apoptosis.[29] *PTEN* acts as a lipid phosphatase that regulates major signal transduction pathways and effectively inhibits phosphatidylinositol-3-kinase (PI3K)-mediated signaling. *PTEN* mutation, which occurs frequently in many solid tumors, is associated with constitutive activation of the PI3K/Akt pathway, resulting in tumors that are generally resistant to apoptosis. In pancreatic cancer, *PTEN* is not mutated but functionally abrogated through loss of expression. It was found that more than 60% to 70% of pancreatic cancer cell lines and tumor tissues have decreased or loss of expression of *PTEN*.[16,30] The role *of PTEN* in pancreatic carcinogenesis was also studied using a pancreas-specific *PTEN* knockout mouse model.[31] Knockout mice display pancreatic ductal metaplasia as the predominant phenotype and occasional PanINs. These lesions are characterized by progressive replacement of the acinar pancreas with highly proliferative ductal structures that contain abundant mucins and express Pdx1 and Hes1, two markers of pancreatic progenitor cells. A fraction of these mice develop ductal malignancy. Further studies showed that ductal metaplasia resulted from the expansion of centroacinar cells rather than transdifferentiation of acinar cells into ductal cells. These results suggest that *PTEN* actively maintains the balance between different cell types in the adult pancreas and that dysregulation of the *PTEN* pathway in centroacinar cells may contribute to the initiation of pancreatic carcinoma in vivo.

Reactivation of Developmental Biology Pathways

Hedgehog, Notch, and Wnt Pathways

The relationship between developmental pathways for pancreatic organogenesis and pancreatic cancer has recently gained in appreciation.[32] The hedgehog (Hh) family of genes—sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh)—encode signaling molecules that regulate multiple functions during organ development and in adult tissues. Hedgehog signaling plays an important role in determining the fate of mesoderm in the primitive gut tube, as well as in early pancreatic development and islet cell function. Multiple groups have reported that the hedgehog, Notch, and Wnt developmental cascades might be reactivated during the development of pancreatic cancer through the upregulated expression and/or activation of these complex signaling pathways.[33] The hedgehog signaling molecules, Shh, Ihh, Ptc, Smo, and Gli1, are frequently overexpressed in pancreatic cancer tissues and cell lines as well as in PanIN lesions.[34,35] Specific inhibition of Hh activity in pancreatic cancer cells using cyclopamine can reduce pancreatic cancer cell growth both in vitro and in vivo (Figure 35-3). The reduction of the proliferative activity of pancreatic cancer cells is mediated through G_0/G_1 cell cycle arrest in vitro or induction of apoptosis in vitro and in vivo. Clinical studies with hedgehog inhibitors in pancreatic cancer are currently in progress. Results from these studies will better define the clinical relevance of this target in pancreatic cancer.

Downstream Events

To elucidate additional genetic alterations that can interact with K-*ras* in pancreatic carcinogenesis, the *Sleeping Beauty*

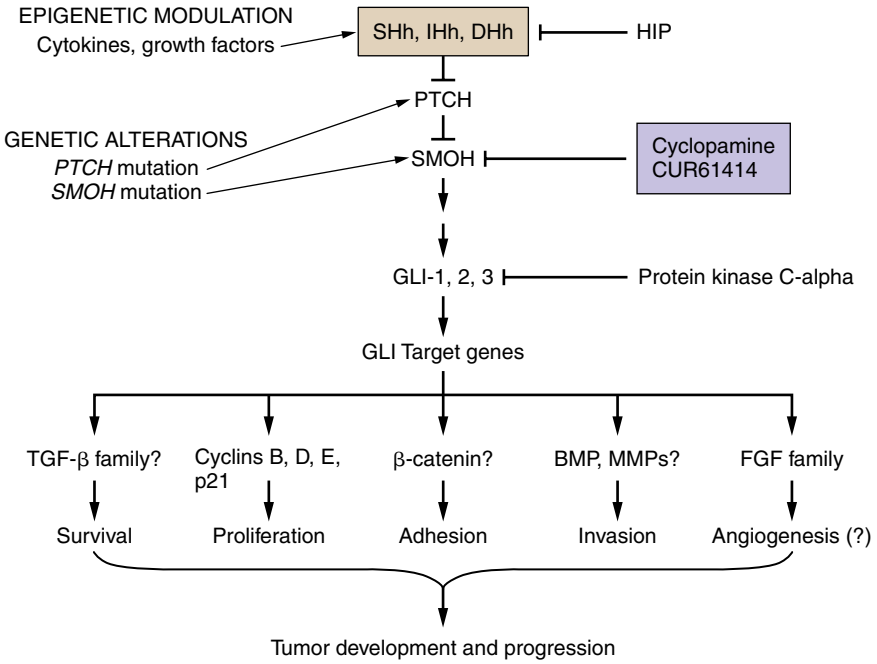


Figure 35-3 Hedgehog signaling, carcinogenesis, and potential therapeutic targets Upregulation of Hh ligands may be mediated by epigenetic events. Mutations in PTCH and SMOH result in activation of hedgehog signaling and are causative in basal cell carcinoma and medulloblastoma. Gli proteins are thought to mediate activation of Hh transcriptional targets potentially important in tumorigenesis, progression, and metastasis. *DHh,* Desert hedgehog; *Gli,* cubitus interruptus-like transcription factor involved in glioma formation; *Hh,* hedgehog; *HIP,* hedgehog interacting protein; *IHh,* Indian hedgehog; *PTCH,* patched; *SHh,* sonic hedgehog; *SMOH,* smoothened. *From Xie K, Abbruzzese JL. Developmental biology informs cancer: the emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers.* Cancer Cell. *2003;4:245-247, with permission.*

transposon system has been recently employed in these K-*ras* mutant mouse models. Mann and colleagues performed a mutation screen using this system and identified 543 potential candidate cancer genes, of which 75 had known mutations in pancreatic cancer.[35] From this, they were able to categorize these genes based on function and clinical outcomes, providing a rich source of information on these potential targets. In a similar experiment, Perez-Mancera and associates used the *Sleeping Beauty* transposon system and identified certain driver mutations that overlapped with those found by Mann and colleagues. These mutations act in concert with K-*ras* to promote progression of pancreatic cancer. Interestingly, introduction of this inducible system also demonstrated a number of candidate genes for the development of pancreatic adenocarcinoma that were unique to this set of experiments.[36] In particular, the X-linked gene deubiquitinase *Usp9x* was inactivated in more than 50% of the tumors. Loss of this gene promoted cellular transformation and correlated clinically with poor prognosis, making it a potential target for future therapeutics.

Desmoplastic Reaction (Tumor Stroma)

One of the morphologic hallmarks of pancreatic adenocarcinoma is its desmoplastic reaction, or tumor stroma. Desmoplastic tissue consists of fibroblasts (the main cellular component), infiltrating inflammatory and immune cells, endothelial cells, and extracellular matrix (ECM) proteins, such as fibronectin and collagen.[37] Pancreatic adenocarcinoma exhibits a threefold increase in interstitial fibrillar collagen (types I and III) compared with the normal pancreas.[38,39] The desmoplastic reaction is also associated with proliferation of fibroblastic cells, which in some cases outnumber tumor cells. Evidence suggests that these are mesenchymal cells, known as stellate cells, which have differentiated into an activated myofibroblastic phenotype. These activated myofibroblasts have been identified as the principal source of type I collagen in the desmoplastic stroma.[40] Pancreatic stellate cells are quiescent and can be identified by the presence of vitamin A–containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, pancreatic stellate cells are transformed from quiescent phenotypes into highly proliferative myofibroblast-like cells that produce ECM proteins. Activated pancreatic stellate cells are observed in abundance in pancreatic tumor tissue, suggesting that they are responsible for the deposition of matrix components and the desmoplastic reaction that surrounds the pancreatic tumor, although pancreatic tumor cells are also capable of producing ECM proteins. Cell culture experiments have demonstrated that pancreatic tumor cells stimulate the growth of pancreatic stellate cells and ECM formation. The growth-stimulating effects are probably mediated by platelet-derived growth factor, fibroblast growth factor 2, and TGF-β1 secreted by pancreatic cancer cells. On the other hand, pancreatic stellate cells can stimulate the growth of pancreatic cancer cells, as demonstrated by an in vivo study in which co-injection of pancreatic stellate cells and tumor cells subcutaneously produced larger and faster growing tumors than injection of pancreatic tumor cells alone. Pathologic examination of tumor tissues showed that an intense desmoplastic reaction in tumors developed after injection of pancreatic stellate cells and tumor cells.[41]

TGF-β is one of the major growth factors stimulating the growth of pancreatic stellate cells. Evidence indicates that the predominant source of TGF-β may be infiltrating granulocytes, although pancreatic tumor cells are capable of producing TGF-β.[42] Immunohistochemical staining of pancreatic tumor tissue showed that isolated cells, mainly located at the invasive edge surrounding cancerous nests, prominently stained for TGF-β. Those cells contain a segmented nucleus and are negative for anti-macrophage (CD68) and positive for anti-granulocyte antibodies, indicating that they are granulocytes.[43]

Hedgehog signaling has also been implicated in the dense stromal reaction associated with pancreatic cancer. A hedgehog paracrine loop between neoplastic cells and stromal cells promotes stromal desmoplasia. The genetically engineered KPC mouse model of pancreatic cancer that closely mimics the tumor-stroma relationship was evaluated to understand the interaction between the hedgehog pathway and the stromal barrier.[44] This model generated poorly vascularized and perfused tumors, similar to those seen in human pancreatic cancer. The researchers showed that drug delivery was impeded in this environment. Furthermore, response to gemcitabine was seen in only a small percentage of the tumors, which closely reflects what is seen clinically with this chemotherapeutic agent. The investigators subsequently demonstrated that a hedgehog pathway inhibitor, IPI-926, could disrupt the stromal barrier and enhance gemcitabine delivery to the tumor, thereby improving cytotoxicity and survival. Although the importance of the stromal barrier in chemoresistance needs to be better understood, stromal cells may be a potential target for future therapies.

Cytokine Production

Pancreatic cancer is known to secrete growth factors that stimulate cancer growth through paracrine or autocrine mechanisms. In addition, pancreatic cancer secretes many cytokines that affect cancer development through interaction with its microenvironment but also have an effect on overall host physiology.[45] Patients with pancreatic carcinoma often have elevated circulating levels of IL-6, IL-10, IL-8, and IL-1RA compared with the levels in healthy individuals.[46] Furthermore, elevation in one cytokine often correlated with elevation in others. For instance, high IL-10 levels were correlated with high IL-8 and high IL-6 levels. It was found that high IL-6 levels in patients with pancreatic carcinoma were correlated with worse survival and weight loss.[47] Further evidence suggested that IL-6 is involved in the development of cachexia, which is a clinical hallmark of pancreatic carcinoma. The pathobiology of cachexia is poorly understood; however, IL-6 and other cytokines may contribute to its development.[48] In one study, gene chip analysis of resected pancreatic cancer tissue including 5600 human genes revealed a significant difference between patients with and without cachexia in only four factors: IL-6, neuropeptide Y Y3 receptor, neurotensin, and islet amyloid polypeptide. IL-6 was significantly overexpressed in pancreatic specimens and elevated in the serum of cachectic patients. A coculture system revealed that pancreatic cancer cells can stimulate IL-6 production exclusively from peripheral blood mononuclear cells derived from cachectic patients, and this effect could be reduced by IL-6–neutralizing antibodies. These data indicate that IL-6 may represent a prominent cachexia-associated factor in pancreatic cancer.

IL-8 expression is also frequently elevated in both serum and pancreatic tumor tissue. IL-8 was originally identified as a neutrophil chemotactic factor. As a member of the CXC chemokine family, IL-8 plays an important role in inflammation and inflammation-induced angiogenesis.[49] It is now known that IL-8 is produced by a variety of normal and tumor cells. It was found that about 80% of pancreatic cancer lines constitutively express high levels of IL-8 in vitro. The role of IL-8 in tumor growth and metastasis has been studied using tumor cell lines, xenograft models, and human tumor tissue.[50] Using orthotopic xenograft models that express different levels of IL-8, it was clearly demonstrated that the level of IL-8 expression correlated with local tumor invasion and distant metastasis. Abrogation of IL-8 expression by antisense oligonucleotides inhibited IL-8 expression and consequently tumor growth and metastasis. In addition, decreased microvascular density of tumor lesions was correlated with decreased levels of IL-8.

Conclusion

Exocrine pancreatic cancer remains a challenging disease. Early diagnosis is infrequent, and therapy has only a limited impact on the survival of patients with advanced pancreatic cancer. Despite these ongoing challenges, our understanding of pancreatic carcinogenesis and the molecular biology of pancreatic cancer has expanded rapidly over the past 5 years. It is anticipated that these advances coupled with the development of biomarkers for early diagnosis will provide the means for early detection of pancreatic cancer and rapidly accelerate the development of effective therapies.

Key References

2. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806.
4. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev*. 2006;20:1218-1249.
8. Tuveson DA, Shaw AT, Willis NA, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*. 2004;5:375-387.
10. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469-483.
14. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1960-1966.
23. Bardeesy N, Aguirre AJ, Chu GC, et al. Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. *Proc Natl Acad Sci U S A*. 2006;103:5947-5952.
33. Sundaram MV. The love-hate relationship between Ras and Notch. *Genes Dev*. 2005;19:1825-1839.
44. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324:1457-1461.
48. Martignoni ME, Kunze P, Hildebrandt W, et al. Role of mononuclear cells and inflammatory cytokines in pancreatic cancer-related cachexia. *Clin Cancer Res*. 2005;11:5802-5808.

References

1. Maitra A, Adsay NV, Argani P, et al. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. *Mod Pathol*. 2003;16:902-912.
2. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806.
3. Iacobuzio-Donahue CA, Fu B, Yachida S, et al. DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. *J Clin Oncol*. 2009;27:1806-1813.
4. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev*. 2006;20:1218-1249.
5. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell*. 1988;53:549-554.
6. Grippo PJ, Nowlin PS, Demeure MJ, Longnecker DS, Sandgren EP. Preinvasive pancreatic neoplasia of ductal phenotype induced by acinar cell targeting of mutant Kras in transgenic mice. *Cancer Res*. 2003;63:2016-2019.
7. Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*. 2003;2:437-450.
8. Tuveson DA, Shaw AT, Willis NA, et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*. 2004;5:375-387.
9. Aguirre AJ, Bardeesy N, Sinha M, et al. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev*. 2003;17:3112-3126.
10. Hingorani SR, Wang L, Multani AS, et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469-483.
11. Ying H, Kimmelman AC, Lyssiotis CA, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell*. 2012;149:656-670.
12. Xiong HQ, Rosenberg A, LoBuglio A, et al. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II trial. *J Clin Oncol*. 2004;22:2610-2616.
13. Pino MS, Shrader M, Baker CH, et al. Transforming growth factor alpha expression drives constitutive epidermal growth factor receptor pathway activation and sensitivity to gefitinib (Iressa) in human pancreatic cancer cell lines. *Cancer Res*. 2006;66:3802-3812.
14. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25:1960-1966.
15. Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. *Clin Cancer Res*. 1999;5:119-127.
16. Ying Y, Elpek KG, Vinjamoori A, et al. PTEN is a major tumor suppressor in pancreatic ductal adenocarcinoma and regulates an NF-kappaB-cytokine network. *Cancer Discov*. 2011;1:158-169.
17. Kouvaraki MA, Korapati AL, Rassidakis GZ, et al. Potential role of Jun activation domain-binding protein 1 as a negative regulator of p27kip1 in pancreatic adenocarcinoma. *Cancer Res*. 2006;66:8581-8589.
18. Abdelrahim M, Smith 3rd R, Burghardt R, Safe S. Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells. *Cancer Res*. 2004;64:6740-6749.
19. Wei D, Wang L, He Y, Xiong HQ, Abbruzzese JL, Xie K. Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of Sp1 transcription factor activity. *Cancer Res*. 2004;64:2030-2038.
20. Wei D, Le X, Zheng L, et al. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene*. 2003;22:319-329.
21. Abdelrahim M, Baker CH, Abbruzzese JL, Safe S. Tolfenamic acid and pancreatic cancer growth, angiogenesis, and Sp protein degradation. *J Natl Cancer Inst*. 2006;98:855-868.
22. Hustinx SR, Leoni LM, Yeo CJ, et al. Concordant loss of MTAP and p16/CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion. *Mod Pathol*. 2005;18:959-963.
23. Bardeesy N, Aguirre AJ, Chu GC, et al. Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. *Proc Natl Acad Sci U S A*. 2006;103:5947-5952.
24. Boschman CR, Stryker S, Reddy JK, Rao MS. Expression of p53 protein in precursor lesions and adenocarcinoma of human pancreas. *Am J Pathol*. 1994;145:1291-1295.
25. Schutte M. DPC4/SMAD4 gene alterations in human cancer, and their functional implications. *Ann Oncol*. 1999;10(suppl 4):56-59.
26. Miyaki M, Kuroki T. Role of Smad4 (DPC4) inactivation in human cancer. *Biochem Biophys Res Commun*. 2003;306:799-804.
27. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res*. 2000;60:2002-2006.
28. Tascilar M, Skinner HG, Rosty C, et al. The SMAD4 protein and prognosis of pancreatic ductal adenocarcinoma. *Clin Cancer Res*. 2001;7:4115-4121.
29. Yamada KM, Araki M. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J Cell Sci*. 2001;114:2375-2382.
30. Asano T, Yao Y, Zhu J, Li D, Abbruzzese JL, Reddy SA. The PI 3-kinase/Akt signaling pathway is activated due to aberrant Pten expression and targets transcription factors NF-kappaB and c-Myc in pancreatic cancer cells. *Oncogene*. 2004;23:8571-8580.
31. Stanger BZ, Stiles B, Lauwers GY, et al. Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell*. 2005;8:185-195.
32. Xie K, Abbruzzese JL. Developmental biology informs cancer: the emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers. *Cancer Cell*. 2003;4:245-247.
33. Sundaram MV. The love-hate relationship between Ras and Notch. *Genes Dev*. 2005;19:1825-1839.
34. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature*. 2003;425:851-856.
35. Mann KM, Ward JM, Yew CC, et al. Sleeping Beauty mutagenesis reveals cooperating mutations and pathways in pancreatic adenocarcinoma. *Proc Natl Acad Sci U S A*. 2012;109:5934-5941.
36. Perez-Mancera PA, Rust AGL, van der Weyden L, et al. The deubiquitinase USP9X suppresses pancreatic ductal adenocarcinoma. *Nature*. 2012;486:266-270.
37. Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer*. 2004;4:839-849.
38. Imamura T, Iguchi H, Manabe T, et al. Quantitative analysis of collagen and collagen subtypes I, III, and V in human pancreatic cancer, tumor-associated chronic pancreatitis, and alcoholic chronic pancreatitis. *Pancreas*. 1995;11:357-364.
39. Mollenhauer J, Roether I, Kern FH. Distribution of extracellular matrix proteins in pancreatic ductal adenocarcinoma and its influence on tumor cell proliferation in vitro. *Pancreas*. 1987;2:14-24.
40. Ottaviano AJ, Sun L, Ananthanarayanan V, Munshi HG. Extracellular matrix-mediated membrane-type 1 matrix metalloproteinase expression in pancreatic ductal cells is regulated by transforming growth factor-beta1. *Cancer Res*. 2006;66:7032-7040.
41. Bachem MG, Schunemann M, Ramadani M, et al. Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology*. 2005;128:907-921.
42. Lohr M, Schmidt C, Ringel J, et al. Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res*. 2001;61:550-555.
43. Aoyagi Y, Oda T, Kinoshita T, et al. Overexpression of TGF-beta by infiltrated granulocytes correlates with the expression of collagen mRNA in pancreatic cancer. *Br J Cancer*. 2004;91:1316-1326.
44. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science*. 2009;324:1457-1461.

45. Moldawer LL, Copeland 3rd EM. Proinflammatory cytokines, nutritional support, and the cachexia syndrome: interactions and therapeutic options. *Cancer*. 1997;79:1828-1839.
46. Ebrahimi B, Tucker SL, Li D, Abbruzzese JL, Kurzrock R. Cytokines in pancreatic carcinoma: correlation with phenotypic characteristics and prognosis. *Cancer*. 2004;101:2727-2736.
47. Bellone G, Smirne C, Mauri FA, et al. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol Immunother*. 2006;55:684-698.
48. Martignoni ME, Kunze P, Hildebrandt W, et al. Role of mononuclear cells and inflammatory cytokines in pancreatic cancer-related cachexia. *Clin Cancer Res*. 2005;11:5802-5808.
49. Xie K, Wei D, Huang S. Transcriptional anti-angiogenesis therapy of human pancreatic cancer. *Cytokine Growth Factor Rev*. 2006;17:147-156.
50. Shi Q, Abbruzzese JL, Huang S, Fidler IJ, Xiong Q, Xie K. Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. *Clin Cancer Res*. 1999;5:3711-3721.

Adrian V. Lee, Steffi Oesterreich, and Nancy E. Davidson

36

The Molecular Biology of Breast Cancer

Molecular Biology of Breast Cancer Initiation and Progression

Normal Breast Development

Studies of breast cancer from the 1970s to the mid-1990s focused mainly on changes in breast cancer with little regard for normal tissue or development. A lack of knowledge of normal mammary gland development and function limited the understanding of tumor-specific changes. In 1998, the NCI-directed Breast Cancer Progress Review Group stated, "Our limited understanding of the biology and developmental genetics of the normal mammary gland is a barrier to progress."[1] This statement led to a major increase in such studies, with mouse models giving invaluable insights into the molecular biology of both normal mammary gland development and breast cancer.[2] Extensive genetic and molecular analysis of mammary gland development in small and large animals has rapidly defined many of the intricate molecular networks, such as interactions between steroid hormone and growth factors, that are critical for all stages of development and function.[3] Intriguingly, many of these same pathways have major roles in breast cancer development and progression and thus are major therapeutic targets.[4] One of the greatest advances has been the recent identification and characterization of mouse mammary stem cells. Sorting cell populations using cell surface markers has shown that the myoepithelial cell layer contains adult mammary stem cells and that a single cell transplanted into the mouse can produce every epithelial cell of the mammary gland.[5,6] Evidence that mammary stem cell number and function are regulated by hormones such as progesterone and RANK ligand is consistent with the major functions of hormones in mammary gland function and may have implications for human breast cancer development and treatment.[7,8] Intriguingly, BRCA1 has also been found to regulate mammary stem cell number and function,[9] and evidence suggests that BRCA1 cancers may arise from a blockade of progenitor cell development.[10]

Clonal and Stem Cell Hypotheses

There are two main models for cancer initiation and progression, the clonal evolution hypothesis[11] and the cancer stem cell (CSC) hypothesis.[12] In the clonal evolution hypothesis, any cell is susceptible to sporadic random mutation, and a particular combination of mutations allows selection of a cell to evolve to become immortal and tumorigenic. Thus any cell within a tumor can maintain tumorigenesis. In contrast, the CSC hypothesis posits that only stem and progenitor cells, which are a minor fraction of cells within a tumor, can give rise to self-renewing tumor cells. These two hypotheses have major implications for understanding breast cancer development and therapeutic intervention.[13] However, the two hypotheses both rely on major assumptions that are virtually impossible to assess, given the inherent difficulty in tracking cell transformation and differentiation in human breast tumors. Although both hypotheses are often presented as competing ideas, it is highly likely that tumorigenesis is a combination of both models.[14] Future studies will be required to define cancer cell growth and differentiation and better define the role of clonal and cancer stem cell function, as this will likely have major implications for the prevention and treatment of the disease.

Preneoplastic Progression

Advances in molecular biology have had a major impact in the understanding of premalignant progression. Early studies using anatomic pathology and epidemiology revealed that certain premalignant breast lesions such as atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) were associated with an increased risk of developing subsequent invasive ductal cancer (IDC).[15] Analysis of changes in DNA copy number and loss of heterozygosity showed that synchronous and metachronous DCIS and IDC showed almost identical genetic changes indicating

that DCIS is the precursor for IDC.[16,17] Consistent with this, the diversity of transcriptomic change and IDC subtypes is similarly found in DCIS.[18] Although 80% to 90% of breast cancer is of ductal origin, a smaller percentage (but still large number of breast cancers) are of lobular origin. These are much less studied; however a similar pattern of progression from atypical lobular hyperplasia to lobular carcinoma in situ to invasive lobular cancer is thought to occur.[19]

Pathophysiology and Risk Factors

Genetics and Family History

Approximately 5% to 10% of breast cancer cases have a familial or hereditary component.[20] Advances in molecular biology have had a profound effect on the diagnosis, risk reduction, and treatment of hereditary breast cancer.[21] Classic genetic mapping and cloning studies identified mutations in two genes, BRCA1 and BRCA2, that account for the majority of hereditary breast cancer. Screening for mutations in BRCA1 and BRCA2 is now standard of care in women with clinical features suggestive of hereditary breast cancer, and recent advances in DNA sequencing permit advanced screening at a reduced price.[22] BRCA1 and BRCA2 are large multifunctional proteins that have a major role in DNA repair.[23] Genetic deletion of these genes in mice and in cells results in genomic instability and sensitivity to transformation.[24] Mutations in other genes such as BRIP1, RAD51, CHEK2, ATM, and PALB2 are also associated with hereditary breast cancer, and importantly, these genes also participate in DNA repair, indicating that loss of DNA repair is a major contributor to familial breast cancer.[23]

The most recent clinically relevant advance to come from understanding the molecular biology of BRCA1 and BRCA2 function has been the finding that cells with mutations in these genes are sensitive to blockade of polyADP ribose polymerase (PARP) and the alternative base-excision DNA repair (BER) pathway.[25] This finding is founded on the genetic precept of synthetic lethality, whereby a cancer cell with wild-type BRCA1 can compensate for inhibition of BER by using the classical homologous recombination pathway. In contrast, cells with loss of BRCA function have no pathway to overcome the loss of BER and thus undergo cell death. The result is a dramatic relative increase in sensitivity to PARP inhibition in cells with loss of BRCA.[26] This concept of synthetic lethality has led to clinical trials showing dramatic responses to PARP inhibitors in cancer patients with germline mutations in BRCA1 or BRCA2.[27]

Hormones

Many risk factors for breast cancer are related to estrogen exposure.[28] These include age at menarche and menopause, parity and age at first-time pregnancy, and breast feeding. The common link among these factors is the number of menstrual cycles that women experience, and thus it is hypothesized that cumulative length of exposure of the breast to sex hormones may increase risk of breast cancer. These data are supported by evidence that surgical or pharmacological suppression of ovarian function significantly reduces breast cancer risk,[29] and prolonged postmenopausal use of combined estrogen and progesterone therapy increases risk.[30]

Environment

Although there are clear geographic differences in the rate of breast cancer, tying these different rates of breast cancer to specific environmental exposures (defined broadly) has been challenging. For example, there is a strong association between risk of breast cancer and fat intake across countries[31]; however, direct proof that fat intake correlates with risk of breast cancer has been challenging to obtain.[32] A recent comprehensive review by the Institute of Medicine reported that alcohol consumption, postmenopausal weight gain, smoking, lack of exercise, and hormone replacement therapy are associated with increased risk of breast cancer.[33] There is also evidence for increased risk with exposure to benzene, 1,3-butadiene, ethylene oxide, chemical pollutants in vehicle exhaust, gasoline fumes, and smoking. Recent studies have begun to link risk factors to specific subtypes of breast cancer and potentially highlight the different etiologies of breast cancer subtypes.[34] A greater understanding of the molecular biology of breast cancer initiation and progression is likely to provide greater clarity about breast cancer risk factors.

Molecular Subtyping

Histopathology and Molecular Pathology

Multiple lines of evidence demonstrate that breast cancer is not a single disease, but a mixture of different subtypes. Within these subtypes, there exists further significant diversity. By histopathology, the majority of breast cancers are invasive ductal cancer (IDC) (about 75%), invasive lobular cancer (ILC) (about 10%), or mixed IDC/ILC (about 5%). Minor populations are mucinous, tubular, medullary, papillary, and metaplastic breast cancers.[19] Histopathology

has been very useful to define these subtypes and then further assess tumor aggressiveness by measures such as tumor grade.[35] Molecular analysis of breast cancers allows further subclassification of the major subtypes, such as IDC, into subtypes with different outcome. The first biomarker, initially discovered and studied more than 40 years ago, was the estrogen receptor alpha (ER). ER-positive breast tumors generally have a somewhat better prognosis, and patients with these tumors are candidates for antihormone therapy.[36] ER-positive tumors can be further subdivided by levels of the estrogen-inducible gene progesterone receptor (PR), with loss of PR indicating lack of ER action and poor outcome.[37] The second subclassification came with the discovery of ErbB2 (HER2) amplification. Approximately 20% of IDC have amplification and overexpression of HER2. Patients with HER2-positive tumors have a poorer natural history, but many respond to anti-HER2 therapy.[38] Tumors that lack expression of ER, PR, or HER2 have been termed *triple-negative breast cancer* (TNBC). There has been intense study of this subset of breast cancer in the past 10 years as they have poor outcome and are insensitive to targeted therapies such as antihormonal or anti-HER2 therapy.[39]

Transcriptomics

The ability to undertake simultaneous measurement of thousands of genes, via microarray technology, allowed a fundamental shift in the study of the molecular biology of breast cancer.[40] The information provided a much finer delineation of breast cancer subtypes than that afforded by histopathology, and also gave insight into the biological underpinning that highlights potential new therapeutic targets. Perou and colleagues performed the first microarray analysis of human breast cancer and identified a set of "intrinsic" genes that defined five major subtypes (luminal A, luminal B, normal-like, HER2-enriched, and basal-like) with different outcomes.[41] These results have remained highly reproducible.[42] A major breakthrough in these studies was the push to make these large datasets publicly available,[43] a move that has greatly facilitated breast cancer research via in silico analysis. Many resources are available for the analysis of large publicly available datasets of breast cancer (e.g., Oncomine.org; gene expression omnibus GEO—www.ncbi.nlm.nih.gov/geo).

From these analyses has come the understanding that luminal tumors express ER and ER-regulated genes. Luminal A tumors tend to have low expression of proliferation genes and have a very good prognosis,[44] whereas Luminal B tumors have lower levels of ER and ER-regulated genes, exhibit markedly higher proliferation and mutation of p53, and have a worse prognosis.[45] Some luminal B tumors are ER+/HER2+. The HER2+ subclass contains tumors that have high levels of HER2 and HER2-regulated genes. A recent integrated analysis of copy number aberrations (CNA) and gene expression showed that the HER2 subtype is largely regulated by the amplification of HER2 (in a cis manner) rather than amplification of other genes that act to increase HER2 expression (in a trans manner).[46]

A large subset (approximately 80%) of TNBC express genes associated with basal/myoepithelial breast cells (such as cytokeratin 5) and has been termed *basal-like breast cancers*. It should be noted that basal-like tumors are not synonymous with TNBC but represent a subset of TNBC.[47]

The normal-like classification of breast cancer is controversial. These tumors account for approximately 5% to 10% of breast cancer and show gene expression profiles similar to normal breast tissue or fibroadenomas such as adipose genes. Many investigators believe that the normal-like subtype is an artifact of low tumor cellularity. Indeed, studies using gene expression profiling of microdissected breast cancer cells do not identify the normal-like subtype.[48] Similarly, gene expression profiling of breast cancer cell lines also does not identify normal-like cell lines.[49] However, the identification of this subtype does highlight the issue of tumor heterogeneity and cellularity when performing microarray analysis of breast cancers. Although tremendous advances have been made in microarray profiling, most of these profiles represent an average of gene expression across multiple cell types within a tumor (including leukocytes, adipose, vascular cells, etc.). It is likely that new approaches, such as single cell transcriptomic profiling,[50] will provide a new level of detailed insight into breast cancer molecular biology.

Although microarray technology allowed genome-wide analysis of mRNA levels, recent advances in massively parallel sequencing of RNA are giving new insight into not only RNA levels, but also changes in RNA splicing and polyadenylation usage[51,52] and noncoding RNAs[53] in breast cancer. In addition, sequencing of two ends of RNA allows the identification of neo-RNA fusion genes that are generated by fusion of two RNAs. A recent comprehensive analysis by paired-end RNA-sequencing of 89 breast cancer cell lines and tumors identified 384 expressed fusion RNAs. However, only one (SEC16A-NOTCH1) was found in more than one tumor.[54] Several genes appeared fused multiple times, but often with different partner genes. Overall this study highlighted the molecular diversity of human breast cancers and the complexity of targeting specific mutations.

Genomics

Genomic instability, the change in DNA structure and copy number, is a hallmark of virtually all breast cancers. Early studies of genomic change relied on cytogenetics and

fluorescence in situ hybridization (FISH) and revealed large chromosome changes and discrete copy number changes such as amplification of HER2. Microarray technology transformed the study of genomics by allowing genome-wide study using comparative genomic hybridization.[55] Genomic instability permits changes in multiple genes required for cancer progression. Consistent with this, analysis of DNA copy number aberrations (CNA) during breast cancer progression shows a large increase in such changes at the transition from ADH to DCIS.[56] Early studies identified recurrent oncogene-containing amplifications such as 8q12 (FGFR1), 8q24 (myc), 11q13 (CCND1), and 17q21 (ErbB2). However these amplified regions often contain numerous genes that may play a role in breast cancer.[56]

Several large studies of CNA in breast cancer have identified three major broad types.[57] The first type, termed *simple*, exhibits few CNA and tends to have gain (1q and 16p) or loss (16q) of whole chromosome arms. This form of CNA tends to be associated with luminal A tumors with good outcome. The second type, termed *amplifier* or *firestorm*, is associated with focal high levels of amplification within a background of other complex gains and losses. A third type, termed *complex* or *sawtooth*, is associated with numerous low-level amplifications and losses across the genome. This pattern is most common in the aggressive TNBC and is associated with TP53 mutation. Many public resources of CNA in breast cancer are available in user-friendly formats such as Tumorscape (www.broadinstitute.org/tumorscape) or the UCSC Cancer genome browser (https://genome-cancer.ucsc.edu).

Several large consortia, including the Cancer Genome Atlas (TCGA), the International Cancer Genome Consortium (ICGC), and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC), are examining genome-wide CNA and gene expression changes in large numbers of breast cancers. METABRIC recently reported a comprehensive analysis of 2136 cases of breast cancer and examined how CNA alters transcriptional profiles, in a cis- or trans-acting manner.[46] Interestingly, they found that the HER2 and basal-like subtypes of breast cancer were most associated with the cis effects of CNA, implying that the genes that undergo CNA themselves tend to be drivers of the diseases rather than the gene products acting in a trans manner to activate other drivers of the disease. Perhaps most striking was the initial observation that an integrated analysis of both CNA and gene expression levels led to the identification of 10 subtypes of breast cancers, each having different outcomes.[46] It will likely be many years before the full implication of datasets such as METABRIC are fully understood and translated into clinical care.

While assays for measuring CNA have rapidly advanced, analysis of structural DNA changes, such as inversions and translocations, has lagged behind. Indeed, high-throughput methods have only recently become available with the advent of massively parallel sequencing.[58] Sequencing of paired ends of DNA allows determination of genomic structure. The initial comprehensive analysis of the MCF-7 cell line revealed numerous structural changes including translocations and novel fusion genes.[59] Paired-end sequencing of other human breast cancers and cell lines unveiled patterns of translocations, and the greatest number of events was seen in aggressive TNBC.[60] Sequencing of cell lines has provided many new insights, one of the most intriguing being chromothripsis,[61] in which cancer genomes seem to have undergone a single catastrophic damaging event with numerous mistakes in the repair, resulting in a large-scale rearrangement of part of the genome. Although it is currently unclear how these complex rearrangements are generated, it is possible that they are due to replication-induced DNA damage and repair.[62]

Determination of somatic base-pair mutations in candidate genes has been undertaken in breast cancers for many years; however, it is only recently that a comprehensive catalog has been obtained via massively parallel sequencing.[63-66] A comprehensive database of somatic mutations in breast cancer can be found at COSMIC, the Catalog of Somatic Mutations in Cancer (sanger.ac.uk/genetics/CGP/cosmic). Similar to other cancers, TP53 is commonly mutated (23% of breast cancers), but the most frequently mutated gene is PIK3CA, the catalytic subunit of phosphatidylinositol-3′-kinase.

Multiplexed Genomic and Transcriptomic Prognostic Tests

A dozen years have passed since the initial description of the five intrinsic subtypes of breast cancers,[41] and many thousands of breast cancers have been subjected to microarray analysis. Although these studies have defined the transcriptional landscape of breast cancer and identified new therapeutic targets,[67] translation of this work to clinical use has been slow.[40] The first multigene assay to be used clinically was OncotypeDx (Genomic Health Inc, Redwood City, Calif). This is a RT-PCR test, based on the measurement of expression of 16 genes and 5 reference genes in ER+ node-negative disease, which can predict risk of recurrence on tamoxifen.[68] More importantly, the test reclassifies standard National Comprehensive Cancer Network guidelines (which place most breast cancer patients at high risk of recurrence) and results in identification of patients with disease with a low risk of recurrence and little benefit from adjuvant chemotherapy. A second microarray-based approach (Mammaprint) for determining breast cancer prognosis is based on the measurement of the expression of 70 genes (Agendia Inc, Irvine, Calif). It classifies breast cancers as either low or high

Table 36-1 Breast Cancer Multiplexed Gene Tests

Test	MapQuantTM	Oncotype Dx®	MammaPrint®	PAM50	Breast Cancer Index	Metastasis Score	Rotterdam Signature	Nuvo Select
Company	Ipsogen Inc	Genomic Health Inc	Agenda BV	Nanostring	bioTheranostics	Celera/ Labcorp	Veridex	Nuvera Biosciences
Method	Microarray	RT-PCR	Microarray	Digital	RT-PCR	RT-PCR	Microarray	Microarray
Sample	Fresh/ frozen	FFPE	Fresh/ frozen	FFPE	FFPE	FFPE	Fresh/ frozen	Fresh/ frozen
No. of genes	97	21	70	50	7	14	76	200
Indication	Any cases	ER + LN –	ER +/– LN –	Any cases	ER + LN –	ER + LN –	ER +	Any cases
Therapy prediction	Chemo	TAM Chemo	TAM Chemo	TAM Chemo	TAM	TAM	TAM	TAM Chemo
FDA status	Unknown	Exempt	IVDMIA 510(k)-cleared	Unknown	Unknown	Unknown	Unknown	Unknown

This table is not comprehensive, but provides an example of breast cancer multiplexed gene tests that are currently in development or have been commercialized. The type of sample, number of genes, indication, and ability to predict therapy may change based on commercial development. Prediction of response to therapy has been shown for many of the tests; however, for some of the tests this may not be used or approved as an indication for the test.
Chemo, Chemotherapy; *ER*, estrogen receptor; *FDA*, U.S. Food and Drug Administration; *FFPE*, formalin-fixed paraffin-embedded; *IVDMIA*, In Vitro Diagnostic Multivariate Index Assay; *RT-PCR*, reverse transcriptase polymerase chain reaction; TAM, tamoxifen.

risk for recurrence[69,70] and predicts for benefit from chemotherapy only in the high-risk group.[70] Both OncotypeDx and Mammaprint assays have been validated only in retrospective analyses and are currently under investigation in prospective clinical trials including TAILORx, RxPONDER, and MINDACT. Biotheranostics has developed a breast cancer index assay based on the measurement of seven genes, which, like OncotypeDx, classifies breast cancers as low, intermediate, or high risk and predicts therapeutic benefit from tamoxifen.[71] Also, the initial "intrinsic gene list" identified by Perou and colleagues has been reduced to a 50-gene test (PAM50), and this test has recently been shown to predict risk of recurrence in ER+ node–negative breast cancer.[72] Examples of multigene tests and their application are given in Table 36-1. More recent studies are focusing less on determining the prognosis of breast cancer and more on developing assays for predicting benefit from therapeutic agents. Thus, there are now "gene expression signatures" of estrogen response, serum action, and IGF signaling, many of which may predict benefit from targeted agents.[73]

Despite extensive analysis of DNA changes (copy number, somatic mutation, and structural change) in breast cancer, relatively few multianalyte tests are currently available.

Molecular Basis of Breast Cancer

ER Action

The steroid hormone estradiol signals through two related receptors, ERα and ERβ.[74] Data from in vitro studies and mouse models, as well as studies using clinical specimens, have provided concrete evidence that ERα is the dominant regulator of both normal breast development[3] and breast cancer.[75] ERα (referred to hereafter as ER) is a nuclear hormone receptor that binds the estradiol with very high affinity. Ligand binding changes receptor conformation to allow binding to enhancer elements in DNA. Initial studies suggested that these DNA elements were close to promoters and provided a simple model of how ER directly affects promoter activity. However, advances in genome-wide DNA binding (e.g., ChIP-seq), chromatin conformation (e.g., Chia-PET), and RNA transcription assays (e.g., GRO-seq) have shown that ER action is much more complicated than previously thought.[76,77] ER often binds hundreds of kilobases upstream of promoters to regulate transcription via looping of large segments of DNA. ER's action is regulated through its interaction with numerous co-regulatory proteins, which can either activate or repress its transcriptional activity.[78] Several of those co-regulators have been shown to be associated with endocrine resistance, such as SRC1,[79,80] SRC3,[81,82] and NCoR1.[83]

Although ER clearly functions as a classical ligand-dependent DNA-binding transcription factor, it may also function in an extranuclear nongenomic manner.[84] This is, at least in part, mediated via growth factor activated signaling pathway, ultimately leading to phosphorylation of ER, especially at S118, S167, and S305, and subsequent recruitment of co-regulators and DNA binding.[85] Although such a role is mechanistically attractive, especially with regard to ER being associated with metastatic processes through interaction with SRC, PI3K, and MAPK signaling, the clinical relevance of ER nongenomic action in breast cancer is controversial. A well-controlled large study using more than

3000 clinical specimens showed that cytoplasmic ER expression occurs at a very low incidence rate of less than 2%.[86] More recent genome-wide studies of ER action are revealing new insight into ligand-independent action. For example, ER ChIP-seq of breast cancer cells treated with EGF identifies an ER cistrome that mediates regulation of genes involved in endocrine resistance in HER2-overexpressing tumors[87] and has also demonstrated critical roles for the chromatin pioneer factor, FoxA1.[88]

Molecular biology studies of the structure and function of ER have had a profound effect on the development and use of anti-ER therapies for breast cancer. The first anti-ER ligand, tamoxifen, was originally developed as a contraceptive but never proved useful.[89] However, several studies showed that tamoxifen was highly successful as a targeted antihormonal therapy for women with all stages of ER-positive breast cancer.[90] Tamoxifen binds the ER, but does not activate gene expression (and indeed it represses many genes). A crystal structure of the ER showed that estradiol binding alters the conformation of the protein to cause movement of helix 12 and allow coactivators to bind ER and increase transcription.[91] Tamoxifen, in contrast, does not cause this molecular switch, in part explaining its antagonistic activity. However, tamoxifen exhibits tissue-specific activity and can be an agonist in tissues such as the uterus and bone, leading to its identification as a selective estrogen receptor modulator (SERM) that exhibits mixed agonist/antagonist activity.[92] Unfortunately, tumors can often exploit the agonist activity of tamoxifen and thus reduce its clinical effectiveness. Many molecular mechanisms for the well-studied area of tamoxifen resistance have been deciphered, with the most studied being increased growth factor signaling.[75]

Another approach to inhibiting ER action is to block production of estradiol via inhibition of the enzyme aromatase in postmenopausal women or suppression of ovarian steroid production (by luteinizing hormone releasing hormones or LHRH agonists) in premenopausal women.[93] The advantage of this method over tamoxifen is that neither aromatase inhibitors nor LHRH agonists show agonist activity. Indeed, aromatase inhibitors have been shown to be superior to tamoxifen for the treatment of early and advanced ER-positive disease in postmenopausal women.[93] Interestingly, however, the metabolism of steroid hormones is complex, and other metabolites may activate the ER to circumvent the loss of activity due to a reduction in estradiol.[94,95] A recent whole-genome sequencing of breast tumors before neoadjuvant aromatase inhibitor therapy has revealed mutations in primary breast cancer that map to several signal transduction pathways, and increased mutation of the TP53 pathway (38%) in aromatase inhibitor–resistant tumors compared to those that responded to therapy (17%).

Perhaps the most logical approach to blockade of ER action in breast cancer would be the total removal of ER protein such that no ligand-dependent or independent activation could occur. ICI 182780 (fulvestrant, AstraZeneca) is a selective estrogen receptor downregulator (SERD) that is similar in structure to estradiol, binds ER with the same affinity, and leads to rapid receptor degradation.[96] The actual mechanism of degradation is unknown but likely involves the proteasome. Clinical development of fulvestrant has been hampered by the fact that it requires regular intramuscular injection, and early trials likely used doses (250 mg) that were not sufficient to saturate ER. A recent Phase II trial comparing first-line fulvestrant (500 mg) to the aromatase inhibitor, anastrozole, showed superiority in time to tumor progression for fulvestrant.[97] Furthermore, anastrozole plus fulvestrant was recently reported to be superior to anastrozole alone.[98] Further delineation of the molecular mechanisms whereby ER activates gene transcription, and how SERMs and SERDs inhibit ER activity, will likely lead to the development of improved anti-ER therapies that minimize the emergence of therapeutic resistance.

Chromatin Remodeling

It is now commonly accepted that epigenetic changes such as DNA methylation, chromatin changes, and regulation of gene expression by miRNA play a role in carcinogenesis in many tumors, including breast cancer.[99] Aberrant DNA methylation has been studied extensively, both at the single gene and genome-wide levels. A number of genes have been reproducibly shown to be methylated in breast tumors, such as RASSF1A, PR, RARβ, CCND2, and BRCA1. However, at this point, no predictive or prognostic marker includes measurement of methylation.[100]

Unexpectedly, sequencing studies of tumors have revealed very frequent somatic mutations in chromatin-modifying genes, including in the family of ATP-dependent chromatin remodeling proteins, enzymes modifying post-translational modification of histones, and histone variants. For example, mutations in the H3K4 methyltransferase MLL are among the most frequent in breast tumors.[63,101] Other histone-modifying enzymes are highly expressed in aggressive breast tumors, such as the H3K27 methyltransferase EZH2, which was also shown to contribute to the expansion of progenitor cells.[102]

It is therefore not surprising that there are many efforts to target deregulated epigenetic pathways in breast cancer. In contrast to hematopoietic malignancies, there are currently no approved breast cancer epigenetic therapies. However, trials are ongoing with drugs that inhibit HDACs and DNA

methyltransferases as well as efforts to target other histone-modifying enzymes, such as EZH2.[99]

Growth Factors

Growth factors play a major role in both mammary gland development and breast cancer and have been studied intensely as therapeutic targets.[103] The best studied growth factor receptor in breast cancer is HER2 (ErbB2). HER2 is amplified in approximately 20% of breast cancers, and its amplification and/or overexpression is associated with poor prognosis.[104] HER2 is a member of the larger HER/ErbB family consisting of epidermal growth factor receptor (EGFR/ErbB1/HER1), ErbB3/HER3, and ErbB4/HER4. Amplification of HER2 is thought to cause increased homo- and heterodimerization with other family members, resulting in constitutive activation of downstream signaling pathways leading to cancer cell growth and survival. The identification of this dominant activating oncogene led to one of the first examples of bedside-to-bench translational research with the development of monoclonal antibodies that block HER2. Trastuzumab (Herceptin, Genentech, South San Francisco, Calif) is a humanized monoclonal antibody that binds the extracellular domain of ErbB2. Trials of trastuzumab plus chemotherapy as first-line therapy in advanced breast cancer improved response rate, time to progression, and overall survival.[105] Similarly, adjuvant use of trastuzumab significantly improves disease-free and overall survival.[106]

Despite major advances in the management of HER2-positive breast cancer with trastuzumab, de novo and acquired resistance is common. This has led to a number of alternative strategies to target ErbB2.[107] Pertuzumab (Perjeta, 2C4; Genentech, South San Francisco, Calif) is a monoclonal antibody that, like trastuzumab, binds the extracellular domain of ErbB2. However, it binds a different part of the domain that is critical for dimerization of ErbB2 to ErbB3. Preclinical and early clinical trials suggest that pertuzumab is active in trastuzumab-resistant breast cancers and can also enhance trastuzumab efficacy when given in combination. This was recently demonstrated in the Phase III CLEOPATRA trial in women with advanced HER2-positive breast cancer[108] and is under further study in the MARIANNE, NEOSPHERE, TRYPHAENA, and APHINITY trials.

Therapeutic drugs targeting the ErbB family tyrosine kinase domains have been developed.[109] Lapatinib (Tykerb, GlaxoSmithKline, London, UK) is a small-molecule reversible inhibitor of both ErbB1 and ErbB2 kinase domains and has been approved for the treatment of HER2+ metastatic breast cancer. Neratinib (HKI-272, Pfizer, New York, NY), in contrast to lapatinib, is an *irreversible* inhibitor of all ErbB kinase domains. Similar to pertuzumab and lapatinib, neratinib has documented activity in trastuzumab-resistant preclinical models and clinical trials and is currently in multiple trials to define its role in the treatment of HER2+ breast cancer.

An alternative and novel approach to strictly targeting HER2 activity is trastuzumab emtansine (T-DM1; Roche, South San Francisco, Calif), a conjugation of an antimicrotubule agent (maytansine) to trastuzumab. Importantly, a comparison of T-DM1 to trastuzumab/docetaxel for first-line treatment of metastatic breast cancer showed both improved response (response rate and investigator-reported progression-free survival) and reduced toxicity for T-DM1.[107]

Growth factors are major regulators of mammary gland development, but they act via an intricate regulation by the steroid hormones estrogen and progesterone.[2] Intriguingly, normal steroid receptor positive mammary epithelial cells do not proliferate in response to steroid hormones, but they send a paracrine signal (most likely IGF and other growth factors) to neighboring cells that then proliferate.[110] This paracrine regulation is thought to be critical for the branching morphogenesis of the developing mammary gland. The intricate interaction between steroid hormones and growth factors is likely one of the first pathways to become dysregulated in tumorigenesis, as transcriptomic analysis of early premalignant lesions found elevation of both ER and growth factor (EGF and IGF) signaling.[111] Crosstalk between steroid hormones and growth factors is apparent not only in normal mammary development, but also in breast carcinogenesis. For the EGFR/ErbB2 pathway, increased hormone signaling is generally associated with reduced signaling. For example, there is a negative correlation between ErbB2 and ER levels, and ER is a repressor of ErbB2 levels via PAX2.[112] In contrast, for the IGF/insulin pathway, ER and PR are both positive regulators, with estrogen in particular upregulating ligand, receptor, and downstream signaling component expression.[113] Although IGF-IR and ER are highly correlated in breast tumors, and thus IGF-IR correlates with good prognosis, recent studies examining IGF-IR specifically in TNBC have shown that it correlates with poor outcome and may be a good therapeutic target.[114]

Experimental evidence from breast cancer cell lines has suggested that a major mechanism of resistance to antihormonal therapy is via upregulation of growth factor receptor pathways.[75] However, until recently, results from clinical trials testing this hypothesis have been disappointing, with relatively little benefit from adding anti-EGFR or anti-IGFR therapies to antihormonal therapy. However, in one promising trial, targeting of a signaling molecule mTOR, which is downstream of both IGF-IR and EGFR, showed that the

combination of an mTOR inhibitor (everolimus) and an aromatase inhibitor was superior to the aromatase inhibitor alone in the treatment of hormone-resistant advanced breast cancer in postmenopausal women.[115]

Angiogenesis

Tumors are generally avascular when they first start to grow; however, as the tumor progresses and increases in size, the distance of cells to blood vessels and nutrients necessitates the new formation of blood vessels (angiogenesis). This angiogenic switch is seen as a critical barrier to tumor growth.[116] Preclinical research has identified many critical factors in the angiogenic switch, and blocking this switch with inhibitors of vascular endothelial growth factor (VEGF) has shown benefit in many preclinical models. Clinical testing of VEGF inhibitors (monoclonal antibodies and tyrosine kinase inhibitors) in addition to chemotherapy for women with advanced breast cancer has shown improvements in progression-free survival but little or no effect on overall survival.[117] Although it was believed that targeting the host blood supply would circumvent cancer cell intrinsic mechanisms of resistance, resistance to VEGF inhibitors is rapid and via multiple mechanisms.[118] Future use of angiogenesis inhibitors in breast cancer will likely require the identification of biomarkers of response to optimize clinical benefit.

Conclusion and Outlook

Investigating the molecular biology of breast cancer has given tremendous insight into the development and evolution of the disease and highlighted pathways for therapeutic intervention. However, as techniques for interrogating the molecular underpinnings of breast cancer have allowed deeper insight, it is clear that the levels of molecular alteration are much greater than previously anticipated. Indeed, although tumors clearly share certain features (such as ER+ and/or ErbB2+), no two tumors are the same, and the difference in their evolution and expansion provides great challenges for targeted therapies. It is anticipated that the next generation of research will likely tackle two main areas: the heterogeneity of molecular alterations in tumors and the clonal origin of breast cancer. Answers to these two questions are likely to have broad implications for the prevention and treatment of breast cancer.

Acknowledgments

This work was supported in part by research grants from the NIH/National Cancer Institute R01CA94118 (AVL), R01097213 (SO), the Breast Cancer Research Foundation (SO and NED), Susan G. Komen for the Cure (AVL), the Pennsylvania Department of Health (AVL and SO), and the Pennsylvania Breast Cancer Coalition (SO).

Key References

2. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer*. 2007;7:659-672.
5. Shackleton M, Vaillant F, Simpson KJ, et al. Generation of a functional mammary gland from a single stem cell. *Nature*. 2006;439:84-88.
6. Stingl J, Eirew P, Ricketson I, et al. Purification and unique properties of mammary epithelial stem cells. *Nature*. 2006;439:993-997.
17. Muggerud AA, Hallett M, Johnsen H, et al. Molecular diversity in ductal carcinoma in situ (DCIS) and early invasive breast cancer. *Mol Oncol*. 2010;4:357-368.
18. Allred DC, Wu Y, Mao S, et al. Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. *Clin Cancer Res*. 2008;14:370-378.
22. Walsh T, Lee MK, Casadei S, et al. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *Proc Natl Acad Sci U S A*. 2010;107:12629-12633.
32. Michels KB, Willett WC. The Women's Health Initiative Randomized Controlled Dietary Modification Trial: a post-mortem. *Breast Cancer Res Treat*. 2009;114:1-6.
33. http://www.iom.edu/~/media/Files/Report%20Files/2011/Breast-Cancer-Environment/BreastCancerReportbrief_2.pdf.
40. Prat A, Ellis MJ, Perou CM. Practical implications of gene-expression-based assays for breast oncologists. *Nat Rev Clin Oncol*. 2012;9:48-57.
42. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003;100:8418-8423.
49. Neve RM, Chin K, Fridlyand J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. 2006;10:515-527.
54. Robinson DR, Kalyana-Sundaram S, Wu YM, et al. Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. *Nat Med*. 2011;17:1646-1651.
60. Stephens PJ, McBride DJ, Lin ML, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature*. 2009;462:1005-1010.
61. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 2011;144:27-40.
63. Ellis MJ, Ding L, Shen D, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature*. 2012;486:353-360.
65. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486:395-399.
81. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst*. 2003;95:353-361.
88. Ross-Innes CS, Stark R, Teschendorff AE, et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature*. 2012;481:389-393.

References

1. http://planning.cancer.gov/library/1998breastcancer.pdf.
2. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer*. 2007;7:659-672.
3. Brisken C, O'Malley B. Hormone action in the mammary gland. *Cold Spring Harb Perspect Biol*. 2010;2:a003178.
4. Nicholson RI, Hutcheson IR, Jones HE, et al. Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. *Rev Endocr Metab Disord*. 2007;8:241-253.
5. Shackleton M, Vaillant F, Simpson KJ, et al. Generation of a functional mammary gland from a single stem cell. *Nature*. 2006;439:84-88.
6. Stingl J, Eirew P, Ricketson I, et al. Purification and unique properties of mammary epithelial stem cells. *Nature*. 2006;439:993-997.
7. Asselin-Labat ML, Vaillant F, Sheridan JM, et al. Control of mammary stem cell function by steroid hormone signalling. *Nature*. 2010;465:798-802.
8. Joshi PA, Jackson HW, Beristain AG, et al. Progesterone induces adult mammary stem cell expansion. *Nature*. 2010;465:803-807.
9. Liu S, Ginestier C, Charafe-Jauffret E, et al. BRCA1 regulates human mammary stem/progenitor cell fate. *Proc Natl Acad Sci U S A*. 2008;105:1680-1685.
10. Molyneux G, Smalley MJ. The cell of origin of BRCA1 mutation-associated breast cancer: a cautionary tale of gene expression profiling. *J Mammary Gland Biol Neoplasia*. 2011;16:51-55.
11. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194:23-28.
12. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414:105-111.
13. Visvader JE. Cells of origin in cancer. *Nature*. 2011;469:314-322.
14. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochim Biophys Acta*. 2010;1805:105-117.
15. Allred DC. Ductal carcinoma in situ: terminology, classification, and natural history. *J Natl Cancer Inst Monogr*. 2010;41:134-138.
16. O'Connell P, Pekkel V, Fuqua SA, et al. Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. *J Natl Cancer Inst*. 1998;90:697-703.
17. Muggerud AA, Hallett M, Johnsen H, et al. Molecular diversity in ductal carcinoma in situ (DCIS) and early invasive breast cancer. *Mol Oncol*. 2010;4:357-368.
18. Allred DC, Wu Y, Mao S, et al. Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. *Clin Cancer Res*. 2008;14:370-378.
19. Bombonati A, Sgroi DC. The molecular pathology of breast cancer progression. *J Pathol*. 2011;223:307-317.
20. Clark AS, Domchek SM. Clinical management of hereditary breast cancer syndromes. *J Mammary Gland Biol Neoplasia*. 2011;16:17-25.
21. Gage M, Wattendorf D, Henry LR. Translational advances regarding hereditary breast cancer syndromes. *J Surg Oncol*. 2012;105:444-451.
22. Walsh T, Lee MK, Casadei S, et al. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *Proc Natl Acad Sci U S A*. 2010;107:12629-12633.
23. Walsh T, King MC. Ten genes for inherited breast cancer. *Cancer Cell*. 2007;11:103-105.
24. Konishi H, Mohseni M, Tamaki A, et al. Mutation of a single allele of the cancer susceptibility gene BRCA1 leads to genomic instability in human breast epithelial cells. *Proc Natl Acad Sci U S A*. 2011;108:17773-17778.
25. Ashworth A, Lord CJ, Reis-Filho JS. Genetic interactions in cancer progression and treatment. *Cell*. 2011;145:30-38.
26. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med*. 2009;361:123-134.
27. Rios J, Puhalla S. PARP inhibitors in breast cancer: BRCA and beyond. *Oncology (Williston Park)*. 2011;25:1014-1025.
28. Lambe M. *Reproductive Factors*. New York, NY: Springer; 2010 119–137.
29. Jakesz R. An update on ovarian suppression/ablation. *Int J Gynecol Cancer*. 2006;16(suppl 2):511-514.
30. Narod SA. Hormone replacement therapy and the risk of breast cancer. *Nat Rev Clin Oncol*. 2011;8:669-676.
31. Prentice RL, Kakar F, Hursting S, et al. Aspects of the rationale for the Women's Health Trial. *J Natl Cancer Inst*. 1988;80:802-814.
32. Michels KB, Willett WC. The Women's Health Initiative Randomized Controlled Dietary Modification Trial: a post-mortem. *Breast Cancer Res Treat*. 2009;114:1-6.
33. http://www.iom.edu/~/media/Files/Report%20Files/2011/Breast-Cancer-Environment/BreastCancerReportbrief_2.pdf.
34. Tamimi RM, Colditz GA, Hazra A, et al. Traditional breast cancer risk factors in relation to molecular subtypes of breast cancer. *Breast Cancer Res Treat*. 2012;131:159-167.
35. Rakha EA, Reis-Filho JS, Baehner F, et al. Breast cancer prognostic classification in the molecular era: the role of histological grade. *Breast Cancer Res*. 2010;12:207.
36. McGuire WL. Steroid receptors in human breast cancer. *Cancer Res*. 1978;38:4289-4291.
37. Cui X, Schiff R, Arpino G, et al. Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy. *J Clin Oncol*. 2005;23:7721-7735.
38. Arteaga CL, Sliwkowski MX, Osborne CK, et al. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat Rev Clin Oncol*. 2012;9:16-32.
39. Rodler E, Korde L, Gralow J. Current treatment options in triple negative breast cancer. *Breast Dis*. 2010;32:99-122.
40. Prat A, Ellis MJ, Perou CM. Practical implications of gene-expression-based assays for breast oncologists. *Nat Rev Clin Oncol*. 2012;9:48-57.
41. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406:747-752.
42. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003;100:8418-8423.
43. Perou CM. Show me the data! *Nat Genet*. 2001;29:373.
44. Geyer FC, Rodrigues DN, Weigelt B, et al. Molecular classification of estrogen receptor-positive/luminal breast cancers. *Adv Anat Pathol*. 2012;19:39-53.
45. Tran B, Bedard PL. Luminal-B breast cancer and novel therapeutic targets. *Breast Cancer Res*. 2011;13:221.
46. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486:346-352.
47. Gusterson BA, Ross DT, Heath VJ, et al. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. *Breast Cancer Res*. 2005;7:143-148.
48. Weigelt B, Mackay A, A'Hern R, et al. Breast cancer molecular profiling with single sample predictors: a retrospective analysis. *Lancet Oncol*. 2010;11:339-349.
49. Neve RM, Chin K, Fridlyand J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*. 2006;10:515-527.
50. Dalerba P, Kalisky T, Sahoo D, et al. Single-cell dissection of transcriptional heterogeneity in human colon tumors. *Nat Biotechnol*. 2011;29:1120-1127.
51. Fu Y, Sun Y, Li Y, et al. Differential genome-wide profiling of tandem 3′ UTRs among human breast cancer and normal cells by high-throughput sequencing. *Genome Res*. 2011;21:741-747.
52. Li G, Bahn JH, Lee JH, et al. Identification of allele-specific alternative mRNA processing via transcriptome sequencing. *Nucleic Acids Res*. 2012;40:e104.
53. Shore AN, Herschkowitz JI, Rosen JM. Noncoding RNAs involved in mammary gland development and tumorigenesis: there's a long way to go. *J Mammary Gland Biol Neoplasia*. 2012;17:43-58.
54. Robinson DR, Kalyana-Sundaram S, Wu YM, et al. Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. *Nat Med*. 2011;17:1646-1651.
55. Climent J, Garcia JL, Mao JH, et al. Characterization of breast cancer by array comparative genomic hybridization. *Biochem Cell Biol*. 2007;85:497-508.
56. Korkola J, Gray JW. Breast cancer genomes—form and function. *Curr Opin Genet Dev*. 2010;20:4-14.
57. Hicks J, Krasnitz A, Lakshmi B, et al. Novel patterns of genome rearrangement and their association with survival in breast cancer. *Genome Res*. 2006;16:1465-1479.

58. Fullwood MJ, Wei CL, Liu ET, et al. Next-generation DNA sequencing of paired-end tags (PET) for transcriptome and genome analyses. *Genome Res*. 2009;19:521-532.
59. Hampton OA, Den Hollander P, Miller CA, et al. A sequence-level map of chromosomal breakpoints in the MCF-7 breast cancer cell line yields insights into the evolution of a cancer genome. *Genome Res*. 2009;19:167-177.
60. Stephens PJ, McBride DJ, Lin ML, et al. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature*. 2009;462:1005-1010.
61. Stephens PJ, Greenman CD, Fu B, et al. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell*. 2011;144:27-40.
62. Liu P, Erez A, Nagamani SC, et al. Chromosome catastrophes involve replication mechanisms generating complex genomic rearrangements. *Cell*. 2011;146:889-903.
63. Ellis MJ, Ding L, Shen D, et al. Whole-genome analysis informs breast cancer response to aromatase inhibition. *Nature*. 2012;486:353-360.
64. Stephens PJ, Tarpey PS, Davies H, et al. The landscape of cancer genes and mutational processes in breast cancer. *Nature*. 2012;486:400-404.
65. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486:395-399.
66. Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature*. 2012;486:405-409.
67. Eroles P, Bosch A, Alejandro Perez-Fidalgo J, et al. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. *Cancer Treat Rev*. 2012;38:698-707.
68. Kim C, Paik S. Gene-expression-based prognostic assays for breast cancer. *Nat Rev Clin Oncol*. 2010;7:340-347.
69. Mook S, Van't Veer LJ, Rutgers EJ, et al. Individualization of therapy using Mammaprint: from development to the MINDACT Trial. *Cancer Genomics Proteomics*. 2007;4:147-155.
70. Knauer M, Mook S, Rutgers EJ, et al. The predictive value of the 70-gene signature for adjuvant chemotherapy in early breast cancer. *Breast Cancer Res Treat*. 2010;120:655-661.
71. Jankowitz RC, Cooper K, Erlander MG, et al. Prognostic utility of the breast cancer index and comparison to Adjuvant! Online in a clinical case series of early breast cancer. *Breast Cancer Res*. 2011;13:R98.
72. Chia SK, Bramwell-Wesley V, Tu D, et al. A 50 gene intrinsic subtype classifier for prognosis and prediction of benefit from adjuvant tamoxifen. *Clin Cancer Res*. 2012;18:4465-4472.
73. Reis-Filho JS, Pusztai L. Gene expression profiling in breast cancer: classification, prognostication, and prediction. *Lancet*. 2011;378:1812-1823.
74. Heldring N, Pike A, Andersson S, et al. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev*. 2007;87:905-931.
75. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med*. 2011;62:233-247.
76. Fullwood MJ, Liu MH, Pan YF, et al. An oestrogen-receptor-alpha-bound human chromatin interactome. *Nature*. 2009;462:58-64.
77. Hah N, Danko CG, Core L, et al. A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell*. 2011;145:622-634.
78. Johnson AB, O'Malley BW. Steroid receptor coactivators 1, 2, and 3: critical regulators of nuclear receptor activity and steroid receptor modulator (SRM)-based cancer therapy. *Mol Cell Endocrinol*. 2012;348:430-439.
79. Myers E, Fleming FJ, Crotty TB, et al. Inverse relationship between ER-beta and SRC-1 predicts outcome in endocrine-resistant breast cancer. *Br J Cancer*. 2004;91:1687-1693.
80. Fleming FJ, Myers E, Kelly G, et al. Expression of SRC-1, AIB1, and PEA3 in HER2 mediated endocrine resistant breast cancer; a predictive role for SRC-1. *J Clin Pathol*. 2004;57:1069-1074.
81. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst*. 2003;95:353-361.
82. Spears M, Oesterreich S, Migliaccio I, et al. The p160 ER co-regulators predict outcome in ER negative breast cancer. *Breast Cancer Res Treat*. 2012;131:463-472.
83. Girault I, Lerebours F, Amarir S, et al. Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. *Clin Cancer Res*. 2003;9:1259-1266.
84. Hammes SR, Levin ER. Minireview: Recent advances in extranuclear steroid receptor actions. *Endocrinology*. 2011;152:4489-4495.
85. International Journal of Breast Cancer, vol. 2011, Article ID 232435, 10 pages, 2011. doi:10.4061/2011/232435
86. Welsh AW, Lannin DR, Young GS, et al. Cytoplasmic estrogen receptor in breast cancer. *Clin Cancer Res*. 2012;18:118-126.
87. Lupien M, Meyer CA, Bailey ST, et al. Growth factor stimulation induces a distinct ER(alpha) cistrome underlying breast cancer endocrine resistance. *Genes Dev*. 2010;24:2219-2227.
88. Ross-Innes CS, Stark R, Teschendorff AE, et al. Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature*. 2012;481:389-393.
89. Jordan VC. Tamoxifen treatment for breast cancer: concept to gold standard. *Oncology (Williston Park)*. 1997;11:7-13.
90. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids*. 2007;72:7-25.
91. Shiau AK, Barstad D, Loria PM, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell*. 1998;95:927-937.
92. Goldstein SR, Siddhanti S, Ciaccia AV, et al. A pharmacological review of selective oestrogen receptor modulators. *Hum Reprod Update*. 2000;6:212-224.
93. Chumsri S, Howes T, Bao T, et al. Aromatase, aromatase inhibitors, and breast cancer. *J Steroid Biochem Mol Biol*. 2011;125:13-22.
94. Sikora MJ, Strumba V, Lippman ME, et al. Mechanisms of estrogen-independent breast cancer growth driven by low estrogen concentrations are unique versus complete estrogen deprivation. *Breast Cancer Res Treat*. 2012;134:1027-1039.
95. Sikora MJ, Cordero KE, Larios JM, et al. The androgen metabolite 5alpha-androstane-3beta,17beta-diol (3betaAdiol) induces breast cancer growth via estrogen receptor: implications for aromatase inhibitor resistance. *Breast Cancer Res Treat*. 2009;115:289-296.
96. Krell J, Januszewski A, Yan K, et al. Role of fulvestrant in the management of postmenopausal breast cancer. *Expert Rev Anticancer Ther*. 2011;11:1641-1652.
97. Robertson JF, Llombart-Cussac A, Rolski J, et al. Activity of fulvestrant 500 mg versus anastrozole 1 mg as first-line treatment for advanced breast cancer: results from the FIRST study. *J Clin Oncol*. 2009;27:4530-4535.
98. Mehta RS, Barlow WE, Albain KS, et al. Combination anastrozole and fulvestrant in metastatic breast cancer. *N Engl J Med*. 2012;367:435-444.
99. Connolly R, Stearns V. Epigenetics as a therapeutic target in breast cancer. *J Mammary Gland Biol Neoplasia*. 2012;17:191-204.
100. Huang Y, Nayak S, Jankowitz R, et al. Epigenetics in breast cancer: what's new? *Breast Cancer Res*. 2011;13:225.
101. Wang XX, Fu L, Li X, et al. Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers. *Pathol Oncol Res*. 2011;17:429-433.
102. Yoo KH, Hennighausen L. EZH2 methyltransferase and H3K27 methylation in breast cancer. *Int J Biol Sci*. 2012;8:59-65.
103. Dickson RB, Lippman ME. Growth factors in breast cancer. *Endocr Rev*. 1995;16:559-589.
104. Gutierrez C, Schiff R. HER2: biology, detection, and clinical implications. *Arch Pathol Lab Med*. 2011;135:55-62.
105. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
106. Yin W, Jiang Y, Shen Z, et al. Trastuzumab in the adjuvant treatment of HER2-positive early breast cancer patients: a meta-analysis of published randomized controlled trials. *PLoS One*. 2011;6:e21030.
107. Verma S, Miles D, Gianni L, et al. EMILIA Study Group. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med*. 2012;367(19):1783-1791.
108. Baselga J, Cortes J, Kim SB, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med*. 2012;366:109-119.
109. Saxena R, Dwivedi A. ErbB family receptor inhibitors as therapeutic agents in breast cancer: current status and future clinical perspective. *Med Res Rev*. 2012;32:166-215.

110. Grimm SL, Seagroves TN, Kabotyanski EB, et al. Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development. *Mol Endocrinol*. 2002;16:2675-2691.
111. Kleinberg DL, Wood TL, Furth PA, et al. Growth hormone and insulin-like growth factor-I in the transition from normal mammary development to preneoplastic mammary lesions. *Endocr Rev*. 2009;30:51-74.
112. Hurtado A, Holmes KA, Geistlinger TR, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature*. 2008;456:663-666.
113. Thorne C, Lee AV. Cross talk between estrogen receptor and IGF signaling in normal mammary gland development and breast cancer. *Breast Dis*. 2003;17:105-114.
114. Litzenburger BC, Creighton CJ, Tsimelzon A, et al. High IGF-IR activity in triple-negative breast cancer cell lines and tumorgrafts correlates with sensitivity to anti-IGF-IR therapy. *Clin Cancer Res*. 2011;17:2314-2327.
115. Baselga J, Campone M, Piccart M, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med*. 2012;366:520-529.
116. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer*. 2003;3:401-410.
117. Mackey JR, Kerbel RS, Gelmon KA, et al. Controlling angiogenesis in breast cancer: a systematic review of anti-angiogenic trials. *Cancer Treat Rev*. 2012;38:673-688.
118. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer*. 2008;8:592-603.

Robert C. Bast, Jr., Ignacio Romero, and Gordon B. Mills

37

Molecular Pathogenesis of Ovarian Cancer

Ovarian cancer is neither a common nor a rare disease. In 2013 in the United States, 22,240 women were diagnosed with ovarian cancer and 14,030 died from this malignancy.[1] The lifetime risk for a woman to develop ovarian cancer is approximately 1 in 70. A fraction of ovarian cancers can arise from germ cells (3%) or from granulosa-theca cells (7%), but approximately 90% of ovarian cancers arise from epithelial cells. Traditionally, epithelial ovarian cancers have been thought to develop from a single layer of flattened cells that cover the ovary or, more frequently, that line cysts immediately beneath the ovarian surface.[2] Neoplasms with similar morphology and behavior can, however, arise from fallopian tube, endometriosis, endosalpingiosis, and the peritoneum.[3] Recent studies have implicated the fimbria of the fallopian tube as the site of origin for as many as 30% of high-grade serous epithelial ovarian cancers, particularly in women with germline mutations of *BRCA1* and *BRCA2*.[4]

Advancing age, an increased number of menstrual cycles, and a positive family history are associated with an increased risk for ovarian cancer, whereas oral contraceptives reduce risk in later life by as much as 50%. Approximately 85% to 90% of epithelial ovarian cancers are sporadic and arise in the absence of a family history of the disease, often associated with spontaneous somatic mutations of *TP53*.[5] Among the 10% to 15% of familial ovarian cancers, germline mutations of *BRCA1* and *BRCA2* are found in the majority, associated with a family history of breast, prostate, and pancreatic cancers. In carriers of germline *BRCA* mutations, TP53 is somatically mutated during malignant transformation and the wild-type BRCA allele is lost, resulting in survival during telomeric crisis, genetic instability, and a homozygous deficiency in homologous DNA repair. Ovarian cancers can also occur in Lynch syndrome families with germline abnormalities in DNA mismatch repair genes, associated with colon and uterine cancers. Rare cases of ovarian cancer are encountered in Li-Fraumeni kindreds with germline mutations of TP53, associated with sarcomas and brain tumors. The lifetime risk of developing ovarian cancer depends on the genetic defect: *BRCA1* (30% to 60%), *BRCA2* (15% to 30%), *HNPCC* (12%), and *TP53* (<1%). Importantly, modifiers of the effects of *BRCA1* and *BRCA2* that can improve the ability to predict risk are being rapidly identified and characterized. Other lower penetrance susceptibility genes such as Rad51C and Rad51D likely contribute to familial predisposition to ovarian cancers.

Epithelial ovarian cancers exhibit a distinctive pattern of progression and metastasis (Figure 37-1). Initially, ovarian cancer cells proliferate within the walls of cysts, invade underlying stroma, and enlarge the ovary, forming a pelvic mass. Although epithelial ovarian cancers can spread hematogenously or through lymphatics, the most frequent route of metastasis is over the surface of the peritoneum. In the absence of anatomic barriers, ovarian cancers that arise from the surface of the ovary or the lining of the fallopian tube can spread through the peritoneal cavity before a palpable mass is formed. Ovarian cancer frequently spreads throughout the pelvis and to the right hemidiaphragm, the bowel mesentery, and the omentum. Multiple nodules of metastatic cancer can stud the peritoneal surface and form dense fibrous adhesions that bind adjacent loops of intestine, producing mechanical obstruction (Figure 37-2). Ovarian cancer can also invade the retroperitoneum, affecting the myenteric plexus and producing paralytic ileus. Intestinal obstruction from either mechanism produces nausea, vomiting, and malnutrition. Ovarian cancer patients generally die from inanition, often complicated by intercurrent infection. As control of intra-abdominal metastasis improves, other sites including the lung and brain are becoming more prevalent.

Another distinctive feature of ovarian cancer is the formation of ascites fluid that contains leukocytes, mesothelial cells, and a varying fraction of tumor cells. Accumulation of ascites fluid produces abdominal distention, which can be the initial symptom of disease. Fluid generally drains from the peritoneal cavity through diaphragmatic lymphatics (see Figure 37-1), which can become occluded by tumor cells, preventing outflow.[6] In addition, tumor angiogenesis produces incompetent vessels that permit greater efflux of proteinaceous fluid from the vascular compartment into the

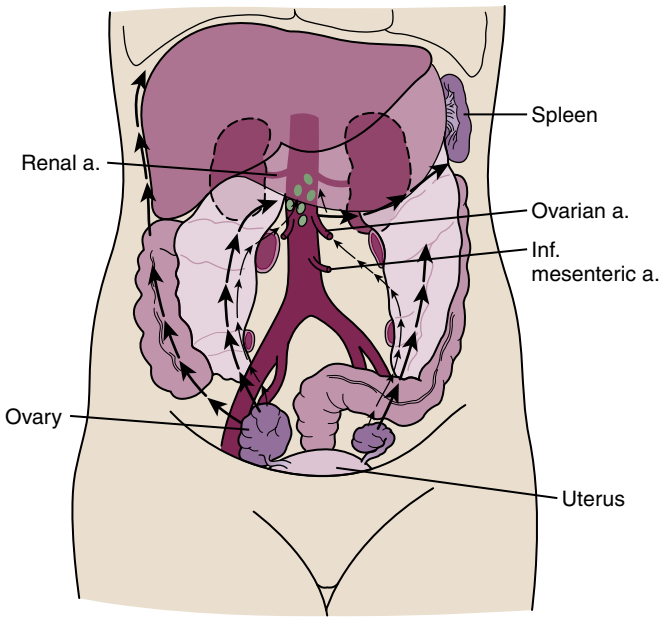


Figure 37-1 Intra-abdominal spread of ovarian cancer. Ovarian cancers metastasize through lymphatics to lymph nodes at the level of the renal hilus, through blood vessels to the liver and other organs, but most frequently over the surface of the parietal and visceral peritoneum from pelvis to diaphragm. *Blast Jr RC, Mills GB. Molecular pathogenesis of epithelial ovarian cancer. In: Mendelsohn J, Howley P, Israel M, Gray JW, Thompson CB, eds. The Molecular Basis of Cancer, 3rd ed. Philadelphia, Pa: Saunders-Elsevier; 2008:441-454.*

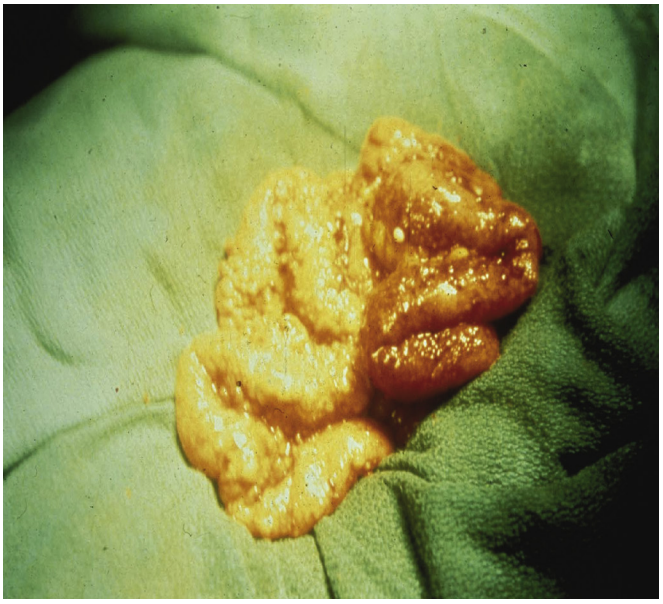


Figure 37-2 Intraperitoneal metastases from epithelial ovarian cancer studding the peritoneal surface.

peritoneal cavity. Ovarian cancer cells can produce copious amounts of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) that is both an angiogenic factor and a permeability-enhancing factor.[7] Neutralization of VEGF with monoclonal antibodies can block ascites formation in murine transplant models[2] and in the clinical setting.[2]

Given the location of the ovaries within the pelvic cavity and the difficulty in assessing abnormalities on routine gynecologic examination, the disease is diagnosed only after it has metastasized in approximately 80% of cases. Ovarian cancer is often described as a "silent killer," but the disease is generally symptomatic, even at early stages in 89% of cases.[8] Symptoms are not, however, specific and are generally attributed to benign gastrointestinal, genitourinary, musculoskeletal, or gynecologic conditions. Detection of a pelvic mass by physical examination or transvaginal sonography generally prompts exploratory surgery to remove the primary tumor and as much of the metastatic disease as possible—so-called cytoreductive surgery. Chemotherapy is generally given for 18 weeks thereafter using a combination of cytotoxic drugs including a taxane (paclitaxel or docetaxel) and a platinum derivative administered intravenously or directly into the peritoneal cavity.

In the 20% of patients with disease that is still localized to the ovaries (stage I), the prognosis is excellent, with up to 90% survival at 5 years using currently available surgery and chemotherapy. As the disease spreads to the other pelvic organs (stage II), to the peritoneal cavity and retroperitoneum (stage III), or to the hepatic parenchyma, pleural cavity, or lymph nodes outside the abdomen (stage IV), the prognosis becomes progressively worse, with a 5-year survival of less than 10% in the last group. Overall, 5-year survival rates have improved significantly ($P < .05$) from 37% in the 1970s to 45% in the 2000s,[2] related in large part to improvements in cytoreductive surgery and combination chemotherapy with carboplatin and paclitaxel. Over the past decade, however, median 5-year survival has not improved for patients with newly diagnosed advanced-stage ovarian cancer treated on clinical protocols of the Gynecologic Oncology Group. Moreover, long-term survival for women with advanced disease has not improved dramatically over the past three decades, and 70% of patients eventually succumb to the disease.

Cellular and Molecular Characteristics of Ovarian Cancer Cells

Heterogeneity of Ovarian Cancers

As in many other malignancies, epithelial ovarian cancer is a clonal disease that arises from a single cell in more than 90% of cases.[9] Despite a clonal origin, epithelial ovarian cancers exhibit marked heterogeneity at a molecular, cellular, and clinical level.

Cell Proliferation

Among cancers from different patients with invasive cancer, the fraction of cycling cells can vary from 1% to 79%

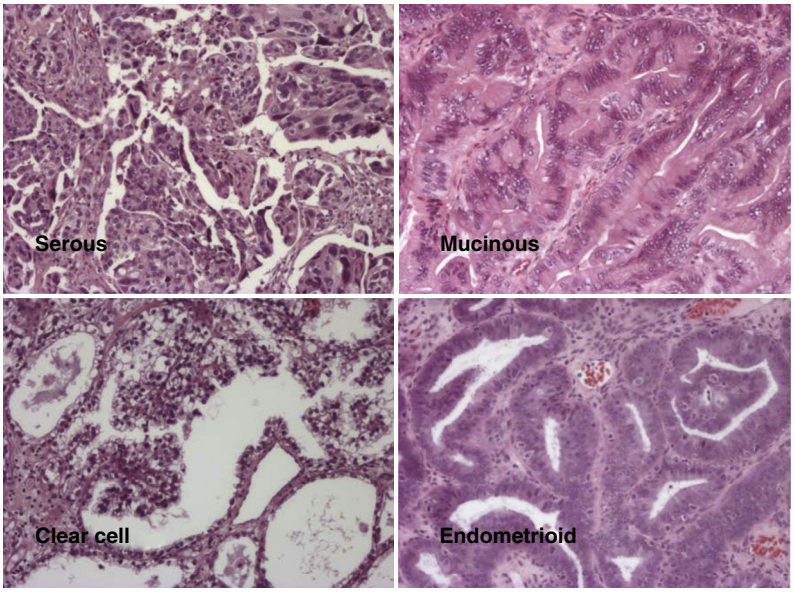


Figure 37-3 Different histotypes of epithelial ovarian cancer.

with a mean of 9% to 34% in different series.[10] Cyclin D1, cyclin E, and CDK2 are upregulated in a minority of cancers with their DNA copy numbers or protein levels correlating inversely with survival. Conversely, the p16, p21, and p27 CDK inhibitors are downregulated or mislocalized in a fraction of cancers, associated with a poorer outcome.

Histotypes

Ovarian cancers exhibit distinct histotypes—serous, endometrioid, clear cell, mucinous—that resemble epithelial components of normal fallopian tube, endometrium, vagina, endocervix, or intestine (Figure 37-3). Histotypes differ with regard to risk factors, genetic abnormalities, expression of tumor markers, and response to chemotherapy.[10] Each histotype exhibits a distinctive pattern of gene expression judged by array analysis, real-time reverse transcriptase–polymerase chain reaction (RT-PCR), and immunohistochemistry.[11] Molecular alterations in ovarian cancers of different histotypes correlate with changes in the normal tissues that they resemble morphologically. The HOX family of homeobox genes plays an important role in determining the histotype of ovarian cancers.[12] During normal development, *HOXA9* is solely expressed in the primordia of the fallopian tubes, *HOXA10* in the developing uterus, *HOXA11* in the lower uterine segment and cervix, and *HOXA13* in the future upper vagina. Expression of these HOX genes is retained in adult tissues but is not observed in ovarian surface epithelia. Expression of *HOXA9, HOXA10,* and *HOXA11* is recapitulated in serous, endometrioid, and mucinous epithelial ovarian cancers, and enforced alterations in expression alter cellular histotype, indicating a causal role.[12]

Low and High Grade (Type I and II)

Low- and high-grade ovarian cancers differ not only in histologic differentiation, but also in pathogenesis, genotype, rate of growth, prognosis, and response to therapy, permitting separation into Type I (low-grade) and Type II (high-grade) lesions.[13-15] Type I cancers include low-grade serous, mucinous, endometrioid, and clear cell histotypes and are often diagnosed in early stage (I or II), grow slowly, and resist conventional chemotherapy. Low-grade tumors frequently express estrogen receptors and may respond to tamoxifen or aromatase inhibitors. The more prevalent Type II cancers include high-grade serous, endometrioid, or undifferentiated histotypes, present at late stage (III or IV), grow aggressively, and respond to conventional chemotherapy, but only occasionally to endocrine therapy. Thus, the distinction between Type I and Type II ovarian cancers can inform choice of treatment.[15,16]

Whereas Type II serous cancers appear to arise de novo from the walls of ovarian cysts or the surfaces of the ovary or fallopian tube, Type I low-grade serous cancers can grow from noninvasive serous "borderline" tumors of low malignant potential in 60% of cases. High-grade Type II ovarian cancers respond to primary chemotherapy with carboplatin and paclitaxel in approximately 70% of cases. Low-grade serous tumors are resistant, but not refractory, to primary platinum-based therapy. Recurrent low-grade serous ovarian cancer has a very low rate of response.[17] Low-grade mucinous and clear cell histotypes respond to conventional chemotherapy in only 26% and 15% of cases, respectively.[2,17]

Low-grade serous cancers exhibit a relatively normal karyotype with wild-type *TP53* and *BRCA1/2*, but exhibit frequent mutations in *KRAS* genes in 19% to 54% of cases. Low-grade serous cancers express the insulin-like growth factor receptor, and the majority overexpress the IGF-1 growth factor, providing a potential target for therapy. Frequent mutations of *KRAS* are found in mucinous cancers and in adjacent borderline tumors, consistent with the mutated gene driving malignant progression. A similar pattern of gene expression has been observed in clear cell and low-grade endometrioid carcinomas, consistent with a common cell of origin.[18] Similar mutations have been found in both histotypes. Inactivating mutations of *ARID1A*, a chromatin remodeling gene, have been reported in 49% of ovarian clear cell carcinomas and 30% of endometrioid ovarian cancers.[19,20] Mutations of *PPP2R1A*, the regulatory subunit of a serine-threonine phosphatase required for chromosome segregation, have been found in 7% of clear cell ovarian cancers.[19] Phosphatidylinositol-3-kinase (PI3K) signaling is activated in low-grade endometrioid cancers through inactivating mutations and epigenetic silencing of *PTEN* and activating mutations of *PIK3CA*. In nonepithelial granulosa cell tumors, recurrent single base mutations (402C-G) of *FOXL2*, a transcription factor implicated in granulosa cell differentiation, have been found in 97% of cases.[21]

Thus, low-grade Type I cancers appear to be driven by mutations that activate Ras/MAP and PI3K signaling in the context of a relatively normal karyotype with wild-type *TP53* and *BRCA1/2*. By contrast, Type II high-grade serous cancers exhibit numerous copy number abnormalities with frequent amplifications and deletions, but with mutations in a very limited number of genes including *TP53* and *BRCA1/2*. The Cancer Genome Atlas Research Network (TCGA) analyzed copy number abnormalities in 489 high-grade serous ovarian cancers, detecting amplification of more than 30 growth stimulatory genes.[22] Amplification and overexpression of genes in the PI3K family occur in more than 50% of Type II cancers, activating the PI3K pathway and conferring "PI3Kness."[23] In the absence of germline abnormalities of *BRCA1/2*, homologous DNA repair can be compromised by somatic mutations of *BRCA1* and *BRCA2* within the cancer alone, BRCA2 can be silenced, and upstream mutations can downregulate BRCA function combined with mutations in other genes potentially involved in homologous recombination, producing "BRCAness" in up to 50% of patients with Type II cancers.[24] DNA sequencing of exons from 316 cancers detected mutations of TP53 in 96%, most of which appeared to be inactivating.[22] Of the 26,000 genes, only a few were mutated in 2% to 4% of cases, including *NF1*, *Rb1*, *BRCA1*, *BRCA2*, and *CDK12*. Unlike Type I cancers, fewer than 1% of Type II cancers had mutations of *BRAF*, *PI3KCA*, *KRAS*, or *NRAS*. Despite the low prevalence of *Rb1* mutations, dysfunction of the Rb pathway was found in 67% of high-grade serous cancers. As in the case of epithelial cancers at other sites, both TP53 and Rb were inactivated in two thirds of Type II ovarian cancers. As indicated earlier, low-grade tumors have a high frequency of mutations in *PIK3CA*, *KRAS*, *ARID1A*, and other putative oncogenes and tumor suppressor genes, whereas high-grade ovarian cancers are characterized by mutations in *TP53* and *BRCA1/2* and marked alterations in DNA copy number. These distinct molecular characteristics indicate that interconversion from Type I to Type II cancers is either a very rare event or does not occur, and thus Type I and Type II tumors likely represent independent diseases. Embracing this concept and performing independent clinical trials and tailored therapy for Type I and Type II tumors will be necessary to improve patient outcomes.

Other Prognostic Subtypes Based on Gene Expression

The pattern of gene expression has been used to identify prognostic subgroups. The Australian Ovarian Cancer Study Group profiled 285 serous and endometrioid tumors to identify six molecular subtypes.[25] Two subtypes were associated with low malignant potential serous tumors and low-grade endometrioid ovarian cancers, whereas the other four transcriptional profiling based subtypes included high-grade serous and endometrioid histotypes including a "mesenchymal" subtype. The immunoreactive subtype expressed T-cell chemokine ligands CXCL11 and CXCL10 and the receptor CXCR3, consistent with a higher level of infiltration by leukocytes. Importantly, lymphocytic tumor infiltration has been associated with an improved outcome. Cases in the proliferative cluster exhibited high expression of the HMGA2 and SOX11 transcription factors, low expression of MUC1 and MUC16 mucins, and high expression of proliferation markers such as MCM2 and PCNA. Differentiated cases have high expression of MUC16, MUC1, and the secretory fallopian tube marker SLPI. Mesenchymal cancers were associated with high expression of HOX genes and markers for stromal components including myofibroblasts (FAP) and microvascular pericytes (ANGPTL2 and ANGPTL1). These subtypes were not associated with changes in overall survival, although a prognostic signature was developed that included 193 genes. Subsequent analyses developed a prognostic "Classification of Ovarian Cancer" (CLOVAR) using gene expression that distinguished groups with markedly different median survival (23 vs. 46 months) and resistance to platinum therapy (63% vs. 23%).[26] Although these classifications can help to stratify future trials, profiles with higher positive and negative predictive value for response to conventional and novel agents will be required in order to affect clinical management.

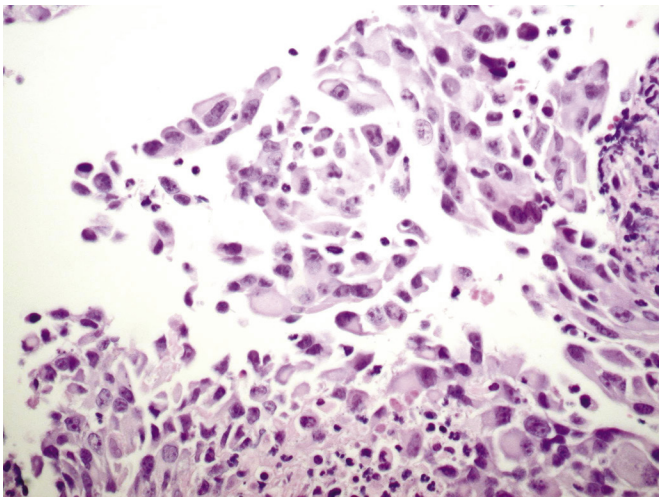


Figure 37-4 Histopathologic features of K-Ras transformed, immortalized human ovarian surface epithelial cells. *With permission from Liu, J, Yang G, Thompson-Lanza JA, et al. A genetically defined model for human ovarian cancer. Cancer Res 2004; 64:1655-1663. PMID: 14996724.*

Immortalization

Telomerase activity is increased in 80% to 90% of ovarian cancers. Substantially greater telomerase activity is found in ovarian cancers than in borderline lesions or normal ovaries. High-grade serous ovarian cancers demonstrate high degrees of genomic instability and copy number abnormalities, possibly related to bridge-fusion breakage that occurs at telomeric crisis, before the upregulation of telomerase. Immortalization of human ovarian epithelial cells can be achieved by the introduction of human telomerase reverse transcriptase (hTERT) after disruption of the TP53 and/or Rb pathways using SV40T/t antigen or siRNA against Rb.[27] Cells immortalized with SV40 T/t and hTERT can be transformed with mutant Ras, producing cancers resembling low-grade human ovarian cancers that grow as nodules on the peritoneum and exhibit serous papillary histology (Figure 37-4).[2] Other combinations of gene aberrations can generate tumorigenic lines from normal ovarian or fallopian tube epithelium, providing a potential approach to explore the roles of specific genomic aberrations in ovarian oncogenesis.

Genomic Abnormalities in Sporadic Ovarian Cancers

A number of genetic and epigenetic abnormalities have been detected in DNA from sporadic ovarian cancers, including amplification, mutation, hypomethylation, chromatin modification, deletion, loss of heterozygosity, and promoter methylation (Table 37-1).[2,14,22] High-grade Type II ovarian cancers are genetically unstable both in terms of DNA copy number and in exhibiting a moderate mutation rate (Figure 37-5). For unknown reasons, areas of genomic aberration are associated with changes in RNA splicing. The *CDK12* gene has been implicated in splicing and is mutated and probably inactivated in 3% of high-grade serous cancers. Aberrant expression of different splicing factors has been observed in ovarian cancer, and altered splicing may affect not only adhesion (*CD44*), but also the activity of putative oncogenes (*EVI-1b* and *AML-1*), as well as the metabolism of xenobiotics (*CYP1A1*) or of multidrug resistance (SPF45).[2] Processing of miRNAs is also impaired in a fraction of ovarian cancers, with decreased levels of Dicer and Drosha in more than half of ovarian cancers.[28] Low Dicer expression was significantly associated with advanced tumor stage (P = .007) and poor survival (P = .02), whereas low Drosha expression was associated with suboptimal surgical cytoreduction (P = .02). Gene silencing with shRNA, but not

Table 37-1 Genetic and Epigenetic Abnormalities in Epithelial Ovarian Cancer[14,22,30]

Activating Events	
Amplification by CGH	1q22 (RAB25), 3q26 (PKCiota, EVI1, PIK3CA), 5q31 (FGF-1), 8q24 (MYC), 19q (PI3Kp85, AKT2), 20p, 20q13.2 (BTAK)
Mutation*	*K-Ras, BRAF, CTNNB1, CDKN2A, PIK3CA, KIT, MADH*
Hypomethylation	*BORIS, CLDN-4, IGF2, MCI, SAT2, SNCG*
Histone modification	*cyclin B1, GATA4, GATA6, p21/WAF1*
miRNA	*BAP1, DLK1, MSX2, PTEN, SIP1, VEGFA, ZEB1/2*
Inactivating Events	
Deletion by CGH	4q, 5q, 16q, 17p, 17q; Xp, Xq
LOH	(>50%): 17p13, 17q21(>30%): 1p, 3p, 5q, 5q, 6q, 7q, 8q, 9p, 10q, 11p, 13q, 18q, 19p, 20; Xp
Mutation	*ARID1A, TP53, Rb1a, APC, BRCA1, BRCA2, CDK12, NF1, PTEN, PPP2R1A*
Promoter methylation	*APC, ARHI, ANGPTL, ARLTS1, BRCA1, DAPK, FBX032, H-CADHERIN, hMLH1, HOXA10, HOXA11, Hsulf-1, ICAM-1, LOT-1, MCJ, MUC2, MYO188, OPCML, PACE-4, PALP-B, PAR-4, PEG3, p16, p21, RASSF1, SOCS1, SOCS2, SPARC, TMS/ASC, TUBB3, 14-3-3σ*
Histone modification	Adam 19, GATA4, GATA6, RASSF1
miRNA	BCL2, FGF2, MMP13, PAR8, c-SRK, VEGFA
Inhibition of growth by chromosome transfer	2, 3, 7, and 22

CGH, Comparative genomic hybridization; *LOH*, loss of heterozygosity; *miRNA*, microRNA.
*www. Sanger.ac.uk.

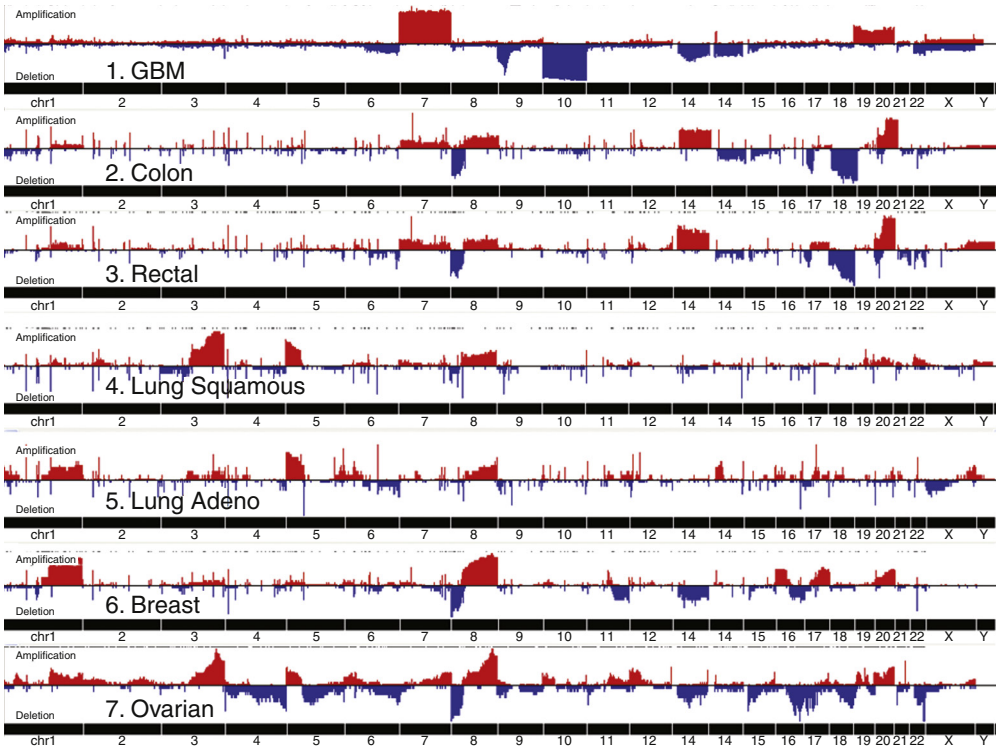


Figure 37-5 Copy number abnormalities in cancers from different sites. *GBM*, Glioblastoma multiforme. *With permission from Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma.* Nature *2011;474:609-615; and Douglas Levine.*

siRNA, may be impaired in cells with low Dicer expression. Mutations of Dicer and Drosha are rare in high-grade serous cancer, but mutations of Dicer have been detected in 29% of nonepithelial ovarian cancers.[29] Abnormalities in miRNAs have been found in many different types of cancers related to gain or loss of DNA copies, methylation, or mutation.[30] In ovarian cancers, copy number abnormalities have been found in 37% of 283 loci known to contain miRNAs.[2] A number of miRNAs may act as tumor suppressors and are downregulated in high-grade serous ovarian cancers, including Let-7a/b/d/f and miRs -15/16, -22, -31, -34a/b/c, -125b, -127-3p, -140, -145, -152, -155, -181a, -199a, and -382.[22,30] Conversely, several miRNAs are upregulated and can promote ovarian oncogenesis or chemoresistance, including miRs -15a/16, -20A, -23a/b, -30a/b/c, -92, -93 and -106a, -135b, -141, -200a/b/c, -244, -299-5p, -302d, and -373. Upregulated miRNAs are being evaluated as biomarkers.

Copy number abnormalities (CNAs) are particularly common in epithelial ovarian cancers. Candidate oncogenes and tumor suppressor genes have been mapped to most but not all of the abnormal sites. Amplification of 1q22 includes the RAB25 oncogene. Among the genes encoded on the 3q26 amplicon, protein kinase C iota (PKCiota), SnoN, MDS1-ecotropic viral integration site-1 (mecom/EVI1), and the p110-α catalytic subunit of phosphoinositide-3-kinase (PIK3CA) are overexpressed in a fraction of ovarian cancers and are associated with a poor prognosis.[2] PKCiota protein is required for the establishment and maintenance of epithelial cell polarity. Levels of aberrant PKCiota are markedly increased and/or mislocalized in the majority of serous ovarian cancers and are associated with increased cyclin E protein expression and proliferation.[2] Cyclin E1 amplification and protein levels correlate with a worsened outcome in ovarian cancer. The FGF-1 peptide growth factor encoded by a gene in the amplicon at 5q31 can stimulate cancer growth, stromal growth, and angiogenesis. An amplicon at 8q24 contains c-myc, which is amplified in up to 40% of ovarian cancers, inducing factors required for proliferation and activating telomerase. Another amplicon on chromosome 19q contains the p85 β subunit of PI3K as well as AKT2, a target of PI3 kinase. Another major amplicon at 20q13.2 contains the *BTAK/Aurora kinase* gene that upregulates c-Myc and activates telomerase.

Loss of tumor suppressor function has been observed in ovarian cancers (Table 37-2). [2,14] In some cases, inactivating mutations have been associated with loss of heterozygosity (LOH) (*BRCA1, BRCA2, TP53, PTEN*), but in others epigenetic changes alone (*RASSF1A, DLEC1*) or in combination with LOH (*ARHI, BRCA1, LOT-1, PEG3, WWOX*) have silenced suppressor function. As described earlier, somatic mutation of *TP53* is observed in nearly all high-grade type II ovarian cancers. Because of the dominant negative activity of some mutant TP53 protein, *TP53* function can be lost with a single genetic event. The pattern of transitions, transversions, and deletions within mutated *TP53* genes resembles the pattern of mutations in factor IX

Table 37-2 Putative Tumor Suppressor Genes in Epithelial Ovarian Cancer[14,22]

Gene	Chromosome	Downregulated or Inactivated	Mechanisms of Downregulation	Function
ARHI (*DIRAS3*)	1p31	60% of all histotypes	Imprinting; LOH; promoter methylation; transcription downregulated by E2F1 and E2F4	26-kDa GTPase; inhibits proliferation and motility; induces autophagy and dormancy; upregulates p21; inhibits cyclin D1, PI3K, Ras-MAP, Stat3
ARID1A	1p35.5	49% of clear cell and 30% of endometrioid histotypes	Mutation	Chromatin remodeling
RASSF1A	3p21		Hypermethylation	Inhibits proliferation and tumorigenicity in many different cancers. Interacts with Ras, inhibiting downregulating cyclin D and signaling through JNK, stabilizes microtubules, regulates spindle checkpoint and fas- and TNF-induced apoptosis
DLEC1	3p22.3	73%	Promoter hypermethylation and histone hypoacetylation	166-kDa cytoplasmic protein that inhibits anchorage dependent growth
SPARC	5q31	70%-90% decreased expression; 9% lost	Transcription, hypermethylation	32-kDa Ca^{2+} binding protein; prevents adhesion
DAB-2 (*DOC2*)	5q13	58%-85% lost	Transcription	105-kDa protein binds GRB2 preventing Ras/MAP activation, prevents c-fos induction, and decreases ILK activity, contributing to anoikis and inhibiting proliferation and anchorage-independent growth and tumorigenicity
LOT-1 (*ZAC1*)	6q25	39%	Imprinting; hypermethylation LOH; transcription downregulated by EGF, TPA	55-kDa nuclear zinc finger protein inhibits proliferation and tumorigenicity
RPS6KA2	6q27	64%	Monoallelic expression in ovary; LOH	90-kDa ribosomal S6 serine threonine kinase that inhibits growth, induces apoptosis, decreases pERK and cyclin D1, increases p21 and p27
PTEN (*MMAC-1*)	10q23	3%-8% mutated; expression lost in 27%, particularly in endometrioid and clear cell histotypes	Promoter methylation; LOH; mutation	PI3 phosphatase; decreases proliferation, migration and survival; decreases cyclin D and increases p27
OPCML	11q25	56%-83%	Promoter methylation; LOH; mutation	GPI-anchored IgLON family member; induces aggregation; inhibits proliferation and tumorigenicity
BRCA2	13q12-13	3%-6%	Mutation; LOH	Binds RAD51 in repair of DNA double strand breaks (DSBs)
ARLTS1	13q14	62%	Promoter methylation	ADP ribosylation factor induces apoptosis
WWOX	16q23	30%-49%, particularly in mucinous and clear cell histotypes	LOH; mutation	Decreases anchorage-independent growth and tumorigenicity; mouse homologue required for apoptosis
TP53	17p13.1	50%-70% overall; 96% of high-grade serous histotype	Mutation	53-kDa nuclear protein induces p21 with cell cycle arrest promoting DNA stability; induces apoptosis
OVCA1	17p13.3	37%	LOH	50-kDa protein; decreases proliferation and clonogenicity; decreased cyclin D1
BRCA1	17q21	6%-8%	Mutation; LOH, promoter methylation	E3 ubiquitin ligase that participates directly in repair of DNA DSBs through homologous recombination; regulates c-Abl; induces *TP53*, androgen receptor, estrogen receptor, and c-Myc
PEG3	19q13	75%	Imprinting; LOH; promoter methylation; transcription	Induces *TP53* dependent apoptosis
PPP2R1A	19q13.44	7% of clear cell histotype	Mutation	Protein phosphatase 2 regulatory subunit inhibits proliferation

Candidate tumor suppressor genes with preliminary reports in the literature also include *APC, BRMS1, CTGF, EPB41L3, MAP2K4, MKK4, RNF43, RP36RA7, PINX1, SFRP4, SLIT2, SOX11, TUSC3, and 53BP1.*
LOH, Loss of heterozygosity.

Table 37-3 Oncogenes Associated with Epithelial Ovarian Cancer[14,22]

Oncogenes	Chromosome	Amplified	Overexpressed	Mutated	Function
Rab25	1q22	54%	80%-89%		Cytoplasmic GTPase/apical vessel trafficking
Evi-1	3q26				Transcription factor
eIF-5A2	3q26	—	—		Elongation factor
PKCi	3q26	44%	78%		Cytoplasmic serine-threonine kinase
PIK3CA (PI3K p110α)	3q26	9%-80%	32%	8%-12%	Cytoplasmic lipid kinase
FGF-1	5q31	—	51%		Growth factor for cancer and angiogenesis
Myc	8q24	20%	41%-66%		Transcription factor
EGFR	7p12	11%-20%	9%-28%	<1%	Tyrosine kinase growth factor receptor
Notch-3	9p13	20%-21%	62%		Cell surface growth factor receptor
K-Ras	12p11-12	5%-53%	30%-52%	2%-24%	Cytoplasmic GTPase
HER-2	17q12-21	6%-11%	4%-12%		Tyrosine kinase growth factor receptor
p85 PI3K	19q				Cytoplasmic lipid kinase
Cyclin E	19q12	12%-53%	42%-63%		Cyclin
AKT2	19q13.2	12%-27%	12%		Cytoplasmic serine-threonine kinase
BTAK/Aurora A	20q13	10%-15%	48%		Nuclear serine-threonine kinase/activates telomerase

Additional targetable genes with low or high gain of copy number in >20% of high-grade serous ovarian cancers include *AKT1, AKT3, CDK2, IL8RB, EPCAM, ERBB3, FGFR2, HDAC4, HSP90AB1, HSP90B1, IGF1, IGFR1, LPAR3, MAP3K6, MAPK15, MAPKAPK2, MAPKAPK5, MECOM, MSTN, MTOR, NCAM1, NOS1, NOS3, PIK3CD, POLB, POLE, RHEB, RICTOR, PPS6KC1, RAPTOR, SKI1, STAT1, STAT4, TERT, TGFB1, TGFB2, TGFBR3, TNFRSF9, VEGFA*.[22]

deficiency (hemophilia B) in the germline that is thought to be related to spontaneous deamination during DNA replication.[5] If spontaneous mutation during proliferation is a critical mechanism driving carcinogenesis, genetic events requiring only a "single hit" may be favored.

Function of imprinted growth regulatory genes can also be lost in a single genetic or epigenetic event. Approximately 70 human genes are imprinted with only one allele expressed at conception, during embryonic development, and in each normal adult cell. Silencing of the maternal or paternal allele is inherited epigenetically. Among the candidate tumor suppressor genes whose function is downregulated in ovarian cancer (see Table 37-2), at least four are imprinted: *ARHI* (*DIRAS3*), *LOT-1*, *NDN*, and *PEG3*. *ARHI* encodes a 26-kDa GTPase that has 50% to 60% homology to Ras and Rap and that is downregulated in more than 60% of ovarian cancers, associated with decreased time to progression.[31] Expression can be downregulated by multiple mechanisms including LOH, methylation and silencing of the functional allele, transcriptional regulation with E2F1 and E2F4, and shortened mRNA half-life.[32] Re-expression of *ARHI* at physiologic levels inhibits clonogenic growth and motility of ovarian cancer cells that lack its expression, inhibiting STAT3 translocation, downregulating cyclin D1 and inducing $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, producing G1 arrest. *ARHI* reexpression decreases levels of HIF1-α and inhibits signaling through Ras/MAP and PI3K/TSC2/mammalian target of rapamycin (mTOR), inducing autophagy and tumor dormancy.[33] Among the other imprinted tumor suppressor genes, *LOT-1* is a zinc finger protein that presumably acts as a transcription factor[2] and *PEG3* is required for TP53-induced apoptosis.[2] Consequently, imprinted genes may regulate the proliferation, motility, and survival of ovarian epithelial cells through multiple mechanisms.

Activation of oncogenes occurs through amplification, overexpression, or mutation in ovarian cancers (Table 37-3).[2,14] The two most common aberrations, mutations of members of the PI3K/AKT pathway and of the Ras/RAF pathway, are discussed in the following sections. The FOXM1 and NOTCH pathways are also aberrant in high-grade serous ovarian cancers, albeit at a lower frequency. Abnormalities of receptor and nonreceptor kinases have also been documented.

In contrast to breast cancer, where HER-2 is amplified and overexpressed in 20% to 30% of cases, HER-2 overexpression was found in only 11% of 837 epithelial ovarian cancers in a Gynecologic Oncology Group trial in which trastuzumab produced objective responses in only 7% of tumors with HER-2 overexpression.[2] Unlike lung cancer, the tyrosine kinase domain of *EGFR* is rarely mutated in ovarian cancer, but the receptor can be amplified in up to 20% of cases. A characteristic constitutively activating deletion in the extracellular domain of EGFR (EGFRvIII), first demonstrated in glioblastomas, has also been detected in a

small fraction of ovarian cancers.[2] Constitutive activation of *EGFR* has, however, not been detected in ovarian cancer cell lines. Inhibition of the EGFR with erlotinib and gefitinib produced only modest clinical activity in ovarian cancer, where objective regression has been observed in 4% to 6% of cases.

Cyclin E maps to an amplicon that is found in a significant minority of ovarian cancers. Knockdown of neighboring genes has shown that *CCNE1* is a driver required for clonogenicity that is associated with chemoresistance.[2] Proteomic studies using reverse phase protein analysis have shown that overexpression of CCNE1 protein marks a distinct cluster of cases with poor prognosis.

BTAK/Aurora A kinase, a serine-threonine kinase required for chromosome segregation and centrosome function, is amplified in 10% to 25% and activated in 48% of ovarian cancers.[2] BTAK/Aurora A kinase regulates telomerase activity. Forced expression of Aurora A induces centrosome amplification, cell cycle progression, and chemoresistance to cisplatin and paclitaxel mediated through AKT in a *TP*53-dependent manner. Chemical inhibition of BTAK/Aurora A kinase downregulates NF-κB, Bcl-XL, and Bcl-2 and enhances sensitivity to chemotherapy. Another cytoplasmic serine-threonine kinase, salt-induced kinase 2 (SIK2), localizes to centrosomes and phosphorylates c-NAP1, permitting centrosome splitting.[34] SIK2 is overexpressed in 30% of ovarian cancers associated with a worse prognosis. Downregulation of SIK2 enhances sensitivity to paclitaxel.

RAB25, a member of the RAB family of small G-proteins implicated in apical vesicle trafficking and polarity, is amplified and overexpressed in approximately half of ovarian cancers and is associated with a poor prognosis. Forced expression of RAB25 protein markedly increased anchorage-dependent and anchorage-independent proliferation; prevented apoptosis and anoikis, including that induced by chemotherapy; and enhanced growth in xenografts.[2] Inhibition of apoptosis was associated with a decrease in the expression of the pro-apoptotic molecules BAK and BAX, as well as activation of the PI3K/AKT pathway. RAB25 is sufficient to increase cellular metabolism and render cells resistant to acute metabolic stress, demonstrating a link between polarity, vesicle trafficking, and cellular metabolism.

In addition to the kinases, growth factors, and signal transducing proteins, members of the family of eukaryotic initiation factors (eIFs) have been implicated in ovarian oncogenesis. The initiation factor eIF-5A2 maps to a region on 3q26 that is amplified in ovarian cancers. Overexpression of eIF-5A2 is associated with advanced-stage ovarian cancer.[2] Forced expression of eIF-5A2 stimulates and antisense inhibits ovarian cancer growth and tumorigenicity.

Several transcription factors are also overexpressed in ovarian cancers. The c-Myc gene is amplified in up to 40% of ovarian cancers, and protein levels are increased in approximately one third of cases, including clear cell and endometrioid histotypes.[2] The c-Myc protein induces E2F1, E2F2, E2F3, and telomerase while blocking TP53-mediated transcription of p21. Despite the probable importance of c-Myc amplification in oncogenesis, no apparent association with survival has been found. A number of approaches to target cells with c-Myc aberrations are beginning to emerge. The association of cMyc with metabolism and particularly in the role of glutamine in cancer biology provides a potential avenue to therapy. The Hippo pathway translational co-activator YAP has also been implicated as a possible oncogene in ovarian cancer that can drive progression and induce resistance to chemotherapy.[35]

Aberrant Signaling

Several tyrosine kinase growth factor receptors can be activated in epithelial ovarian cancers, including EGFR, HER-2/HER-3, M-CSFR, IGFRI, FGFR4, and PDGFR. Binding of relevant ligands to one or more of these receptors can activate signaling through Ras/MAP, PI3K, JNK, JAK/STAT, PKC, and PLCγ or PLC-*x* with a concomitant alteration in calcium fluxes. Among these several signaling pathways, the Ras/MAP and PI3K pathways appear to be particularly important in regulating the growth, survival, metastatic potential, and drug resistance of ovarian cancer cells. Although the RAS/MAPK and PI3K pathways exhibit few mutations in high-grade serous ovarian cancers, the pathways are frequently activated. The PI3K pathway may be activated by changes in copy number of multiple pathway components as well as by loss of PTEN and INPP4B. Indeed, in the TCGA dataset, the RB1 pathway was dysregulated in 67% of high-grade serous cancers and the PI3K pathway in 45%.[22] The NOTCH signaling pathway was dysregulated in 22%. Stimulation of FGFR4 by FGF-1 activates mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), and the WNT signaling pathways.[36]

In many tumor types, K- or H-Ras is activated by mutations at codons 12 or 61, permitting constitutive binding of GTP rather than GDP to the Ras protein. Activated Ras can signal through the MAPK, PI3K, or Ral pathways, affecting proliferation, survival, motility, invasion, and drug resistance. As noted earlier, the *Ras* gene is mutated in approximately half of Type I low-grade serous cancers, but rarely in Type II high-grade cancers. Nevertheless, Ras activity as assessed by GTP binding is increased in many Type II high-grade cancers.

The PI3K pathway regulates survival, proliferation, motility, angiogenesis, glucose metabolism, and drug resistance. Activation of the PI3K pathway is observed in 50% of ovarian cancers through multiple mechanisms including amplification or activating mutation of the PI3K p110

α, activating mutation of the PI3K regulatory subunit p85, inactivating mutation of the PTEN phosphatase and amplification of AKT2 (see Table 37-3).[2] The p110 subunit of PI3K is amplified in 9% to 22% of ovarian cancers, overexpressed in 32%, and mutated in 9% to 12%, predominantly in low grade.[2] The pattern of PIK3CA mutation is histotype dependent, being common in endometrioid and clear cell tumors, but rare in serous cancers. The AKT serine-threonine kinase is generally activated physiologically by the products of PI3K. However, in 12% to 27% of ovarian cancers, the *AKT2* kinase gene is amplified and is associated with an increase in AKT kinase activity.[2] The p70S6 kinase is downstream of both PI3K and AKT kinase in the signaling cascade. The p70S6 kinase may have a critical role in modulating drug resistance in that rapamycin, an inhibitor of p70S6 kinase, can potentiate sensitivity of some ovarian cancer cell lines to cisplatin-induced apoptosis.[2] Inhibition of activated PI3K decreases cell growth, induces apoptosis, and potentiates paclitaxel chemotherapy. The PI3K pathway is being explored as a therapeutic target in many cancer lineages, including ovarian cancer.

Normal ovarian surface epithelium expresses small amounts of macrophage colony stimulating factor (M-CSF, CSF-1), but little if any of fms, the tyrosine kinase receptor for this ligand. At least 70% of ovarian cancers express and secrete substantially greater amounts of M-CSF, and approximately 50% of cancers express the fms receptor.[2] Interaction of M-CSF with fms stimulates invasiveness and upregulates urokinase-like plasminogen activator (uPA). Expression of uPA correlates with tumorigenicity of ovarian cancer cell lines in xenograft models.[2]

Among the nonreceptor tyrosine kinases, the Src tyrosine kinase can be physiologically activated in ovarian cancer cell lines, but the *Src* gene is rarely amplified or mutated in surgical specimens.[2] Stat3 is phosphorylated by the Janus kinase (JAK) and Src kinase. Phosphorylated Stat3 forms dimers that can be translocated to the nucleus, binding DNA and inducing transcription of genes required for proliferation (*cyclin D*, *c-Myc*, *c-Fos*), survival (*Bcl-2*, *Bcl-XL*, *XIAP*, *survivin*), and angiogenesis (*VEGF*). Stat3 can be activated on the intracellular domains of peptide growth factor receptors (EGFR) and cytokine receptors (IL-6R) or by direct interaction with either Src or Abl. Activated pStat3 has been detected in 86% of 322 ovarian cancers.[2] Nuclear localization of pStat3 has been observed in 71% of cases, associated with decreased overall survival. Autocrine and paracrine stimulation with IL-6 activates Stat3, increasing both proliferation and motility. Inhibition of JAK inhibits IL-6–stimulated chemotaxis toward serum and haptotaxis toward fibronectin.[2] Knockdown of Stat3 with siRNA inhibits motility and prevents translocation of Stat to focal adhesions. The imprinted tumor suppressor gene *ARHI* inhibits proliferation and motility, binds to Stat3, and sequesters it in the cytoplasm, preventing translocation to the nucleus and to focal adhesions.[2] In addition, *ARHI* can prevent binding of Stat3 to DNA Stat Response Elements in promoter regions.

The endothelin A peptides (ET-1, ET-2, and ET-3) are potent mitogens for several human tumors. ET-1 and its ETA receptor (ETAR) are overexpressed in primary and metastatic ovarian cancers, providing the potential for autocrine stimulation.[2] Interaction with ET-1 also transactivates EGFR, stimulates proliferation, blocks apoptosis, activates integrin-like kinase (ILK), upregulates matrix metalloproteinases (MMPs), and increases vascular endothelial growth factor (VEGF) expression, enhancing angiogenesis.

Lysophosphatidic acid (LPA) is produced constitutively by mesothelial cells and some ovarian cancer cells and accumulates at high levels in the ascites of nearly all ovarian cancer patients.[37] LPA can be detected in the plasma of most patients at all stages of disease. The LPA-2 and LPA-3 receptors are markedly upregulated in ovarian cancers.[2] Interacting with these receptors, LPA stimulates calcium influx, proliferation, motility, chemotaxis, invasion, and resistance to chemotherapeutic agents, signaling through the RAS/MAP and PI3K pathways. LPA is a potent inducer of VEGF, IL8, IL6, and gro, implicating LPA in the accumulation of ascites, neovascularization, and metastasis. LPA can cross activate the EGFR receptor family and other receptor tyrosine kinases, potentially contributing to the activity of these receptors in ovarian cancer. Both the enzyme producing LPA, autotaxin, and the enzymes degrading LPA (LPPs) are aberrant in ovarian cancer. Drugs targeting LPA production and action are potent inhibitors of metastasis.

All three TGF-β isoforms are expressed by normal ovarian surface epithelial cells and regularly inhibit their proliferation, maintaining autocrine growth inhibition.[2] Loss of expression of TGF-β or loss of responsiveness to the growth inhibitory factor is detectable in a fraction of ovarian cancers. Moreover, TGF-β can stimulate the motility and invasiveness of transformed cells. Although mutation in Smad4 is observed in a fraction of ovarian cancers, both TGF-βRI and TGF-βRII receptors are generally intact, as is Smad signaling.[2] Loss of growth inhibition and increased invasiveness may relate to *EVI-1* overexpression that is observed in 43% of ovarian cancers[2] and is thought to inhibit transcription of TGF-β–responsive genes. SnoN and AML1, which bind to and regulate Smads, are also aberrant in ovarian cancer.

Müllerian inhibition substance (MIS) bears homology to TGF-β, binds to a receptor with similar structure and function, and is produced by the Sertoli cells of the testis and granulosa cells of the ovary.[2] During embryonic development of gonadal structures, MIS induces atrophy of the Müllerian duct in male mammals. MIS inhibits growth of human

epithelial ovarian cancer cells in culture and in xenografts. Binding of MIS to MISII G-protein–coupled receptors upregulates p16, produces G_1 cell cycle arrest through an Rb-independent mechanism, and induces apoptosis. Some 56% of human ovarian cancers express MISII receptors, and clonogenic growth can be inhibited with MIS in more than 80% of cancers bearing receptors. MIS enhanced the anticancer activity of suboptimal doses of chemotherapeutic agents against human and murine ovarian cancer cell lines in culture and as xenografts.[2]

Stem Cells

Both normal and malignant tissues are thought to contain small subpopulations of stem cells with unlimited replicative potential that can be passed serially from mouse to mouse in vivo or as spheroids in cell culture. In cancers, tumor-initiating cells that have stem cell–like characteristics are generally resistant to chemotherapy and radiotherapy. The phenotype of human ovarian cancer stem cells remains to be fully defined. Increased tumor-initiating potential has been associated with $CD133^+ALDH1^+$ and $CD44^+CD117^+$ subpopulations in ovarian cancer cell lines and clinical specimens.[38] CD133 has been associated with stem cells from normal and malignant tissues arising at multiple sites. Aldehyde dehydrogenase mediates resistance to certain drugs and toxins. CD44 is the hyaluronate receptor required for adhesion, and CD117 is the transmembrane tyrosine kinase growth factor receptor, c-Kit. CD24 can be associated with both subpopulations and marks ovarian cancer cells with tumor-initiating capacity and chemoresistance. $CD24^+$ ovarian cancer cells express a number of stem cell biomarkers including Nestin, Beta-catenin, Bmi-1, Oct3/4, Notch1, and Notch4. Recent studies suggest that normal ovarian stem cells occupy a niche in the hilus of the mouse ovary at the junction between the ovarian surface epithelium, the peritoneal mesothelium, and the tubal (oviductal) epithelium. The cells cycle slowly and express a number of stem cell markers including ALDH1, LGR5, LEF1, CD133, and CK6B. Spheroids can be passaged serially in culture, and cells can be transformed by inactivating *TP53* and *Rb1*.[39]

Animal Models

Among the animal models, spontaneous ovarian cancers occur in approximately 40% of egg-laying hens 4 years of age.[40] Some 46% of ovarian cancers have mutations of *TP53*, and the majority express MUC16. Chickens have been used to test different hormonal strategies for preventing epithelial ovarian cancer.

Murine ovarian epithelial cells have been engineered to express different combinations of oncogenes and tumor suppressors.[40] When target cells were derived from transgenic mice that lacked TP53 expression, the addition of any two of three oncogenes—c-Myc, activated K-Ras, and activated AKT—were sufficient to induce high-grade cancer in ovarian surface epithelial cells. In mice that were deficient for both *TP53* and *BRCA1*, *Myc* overexpression is sufficient to induce cancers. In a serous murine ovarian cancer model, disruption of either *Rb* or *TP53* produced relatively few ovarian cancers, whereas disruption of both *Rb* and *TP53* produced a greater number of high-grade epithelial ovarian cancers, albeit with long mean latency (227 days). High-grade ovarian epithelial cancers were induced in a third model when SV40 T antigen, which blocks both *TP53* and *Rb*, was driven by the Müllerian hormone type 2 receptor (*Ambr2*) promoter. A model for low-grade ovarian cancer has been generated in mice in which the *PTEN* gene was disrupted and an oncogenic form of *KRasG12D* was expressed selectively in ovarian surface epithelial cells, using mice in which Cre recombinase was driven by the *Ambr2* promoter. Finally, high-grade serous cancers of the fallopian tube have developed in in mice where *PTEN* and *Dicer* were conditionally depleted using the *Ambr2-Cre* mouse strain. Consequently, ovarian cancers have been associated with loss of TP53, Rb, PTEN, and Dicer function, as well as activation of Ras, AKT, and Myc. To date, however, murine models have not succeeded in mimicking the abnormal DNA copy numbers observed in human ovarian cancers, possibly related to the telomere length in murine cells and relative resistance to telomeric crisis.

Interaction of Ovarian Cancer Cells with the Microenvironment

Loss of Adhesion, Epithelial-Mesenchymal Transition, Invasion, and Metastasis

Several molecular alterations contribute to the distinctive pattern of metastasis observed in ovarian cancer. For those cancers that arise from the surface of the ovary or fimbriae of the fallopian tube, cancer cells must dissociate from the basement membrane and then survive anoikis within the peritoneal cavity. Whereas normal ovarian surface epithelial cells bind to both laminin and to collagen in the basement membrane, loss of adhesion to laminin occurs following malignant transformation.[2] In the normal ovary, the $\alpha_6\beta_4$ integrin laminin receptor is detected over the entire basal surface of epithelial cells at points of contact with the basement membrane, whereas solid ovarian cancers exhibit only focal expression of this integrin. Ascites tumor cells have markedly

decreased expression of α_6 and β_4, consistent with the possibility that downregulation of integrin expression releases tumor cells from the basement membrane. Transformed cells can dissociate individually or as multicellular aggregates or spheroids that are carried passively by peritoneal fluid to sites of metastasis in the peritoneum and omentum. Interaction of integrins with other cancer cells in aggregates can regulate activation of FAK, ILK, PI3K, and AKT. Signaling through these pathways can determine whether cancer cells undergo apoptosis or anoikis on dissociation from the substratum. In addition to changes in integrin expression, alterations occur in the cadherins that regulate adhesion between epithelial cells. Normal surface epithelial cells of the ovary and fallopian tube have low levels of E-cadherin and may depend on N-cadherin to maintain association between epithelial cells.[41,42] In contrast to cancers that arise from many other organs, E-cadherin levels can actually increase during malignant transformation as ovarian cancers differentiate into multiple histotypes, but only low levels of E-cadherin are found in poorly differentiated ascites cells where N- and P-cadherin are often upregulated.

Epithelial-to-mesenchymal transition (EMT) occurs during metastasis in many carcinomas, including those that arise in the ovary. Increased expression of the transcription factors Slug and Snail are associated with loss of intercellular adhesion, as well as specific repression of adherens junction components (E-cadherin and β-catenin), tight junction components (occludin and ZO-1), desmosomal junction components (Dsg2), and neutrophil gelatinase-associated lipocalin (NGAL).[2] However, as noted earlier, the histologic differentiation of ovarian cancer cells potentially driven by homeobox genes can override the effects of EMT and result in increased E-cadherin levels. N-cadherin and vimentin are increased and Rac1, Rho A, and cdc42 GTPases are activated, as ovarian cancer cells assume a spindle shape and become more motile. EMT of ovarian cancer cells can be driven by endothelin A, EGF, LPA, Rab25, bone morphogenic protein-4 (BMP4), hypoxia, and 17β-estradiol. EMT can facilitate invasion of basement membrane and local stroma for those epithelial ovarian cancers that arise in cysts.

To implant on the peritoneal surface, ovarian cancer cells must attach to mesothelial cells through β1 integrins, CD44, and MUC16. β1 integrins on the cancer cell surface bind to VCAM-1, fibronectin, laminin, and type IV collagen on mesothelial cells and to type I and III collagens on the underlying basement membrane.[2,43] Antibodies that block anti-β1 integrin or matrix proteins can prevent adherence of some, but not all, ovarian cancer cell lines to mesothelial monolayers.

Mesothelial cells also express hyaluronic acid, to which the CD44 hyaluronate receptor can bind. Whereas normal ovarian cells express the canonical CD44S receptor, more than 70% of ovarian cancers exhibit a diverse mixture of CD44 splice variants.[2] Anti-CD44 antibodies can partially block adhesion of ovarian cancer cells to peritoneal mesothelial cells and can reduce the frequency of peritoneal metastases.[2] Ezrin, part of the submembrane linking complex that connects CD44 to the cytoskeleton, is strongly expressed in 49% of ovarian cancers and is associated with reduced overall survival.[2] Knockdown of ezrin with siRNA inhibits invasiveness. Interestingly, ascites tumor cells have decreased CD44 expression.

MUC16 on the surface of ovarian cancer cells can bind to mesothelin, a glycosylphosphatidylinositol-anchored glycoprotein expressed on the surface of mesothelial cells. MUC16, expressed by 80% of ovarian cancers, is a high-molecular-weight (more than 1 million Da), highly glycosylated mucin with a cytoplasmic tail. The extracellular domain of MUC16 contains at least 40 repeating subunits of 154 amino acids.[44] MUC16 peptides dock with mesothelin, and binding of ovarian cancer cells to mesothelial cells can be blocked with anti-mesothelin antibodies.[2] Interaction appears to depend on interaction with N-glycans associated with MUC16. Knockdown of MUC16 decreases the invasiveness of ovarian cancers.[2]

Shed MUC 16 (CA125) has provided a serum biomarker for monitoring the course of ovarian cancer during treatment.[45] Increases or decreases in CA125 have correlated with disease course in more than 80% of patients with elevated serum levels of the marker. CA125 has been used to distinguish malignant from benign pelvic masses, identify persistent disease, and detect disease recurrence.[46] Although individual values of CA125 or annual transvaginal sonography (TVS) have not been sufficiently sensitive or specific for early detection of ovarian cancer,[47] sequential two-step strategies have demonstrated greater specificity where rising CA125 identifies a small fraction of patients who would benefit from TVS.[48] Greater sensitivity can be attained with multiple biomarkers.[49]

Degradation of basement membrane at the site of local invasion and in metastases requires upregulation of type 1-MMP, MMP-2, MMP-9, uPA, and kallikrein activity, resembling changes during ovulation. The ability of tumor cells to migrate and to invade matrigel membranes is increased by VEGF, EGF, heregulin, TGF-β, BMP4, hepatocyte growth factor, M-CSF, TNF-α, heregulin, and LPA. Signaling through Ras/MAP, PI3K/AKT/p70S6K, Src and Stat has been implicated in migration and invasion. Recent work points to the importance of fibronectin-mediated activation of α5β1 integrin, which signals through c-Met, Src, and FAK to stimulate invasion and metastasis.[50] Effector proteases, including MMP2, MMP7, MMP9, IGFBP2, uPA, and the kallikreins, have all been associated with ovarian cancer cells in culture and in pathologic

specimens. MMP-2 and MMP-9 are associated with very early dysplastic lesions where basement membrane is breaking down.[2] Ovarian stromal cells are an important source for several of the proteases, including MMP-9, which has been associated with infiltrating monocytes.

The human kallikreins (hKs) include some 15 different serine proteases with a high degree of homology that map to a cluster on chromosome 19q13.4.[2] Twelve are transcriptionally upregulated in ovarian cancer. In aggregate, the kallikreins degrade multiple matrix components including fibronectin, vitronectin, laminin, and collagen I, II, III, and IV. Transfection of hK4, hK5, hK6, and hK7 does not affect proliferation, but increases invasiveness in vitro and formation of peritoneal metastasis in *nu/nu* mice. Kallikrein activity is inhibited physiologically by serpins and antithrombin-3. Several kallikreins are being evaluated as biomarkers for detection or prognostication in ovarian cancer where hK5, hK6, hK7, hK8, hK10, and hK11 are upregulated and hK14 downregulated in tissue and in serum. Elevated hK5, hK6, hK7, and hK15 have been associated with a poor prognosis and elevated hK8 and hK9 with a good prognosis.

Binding of cancer-associated integrins to extracellular matrix can stimulate chemotaxis and invasion. Adhesion of ovarian cancer cells to collagen and clustering of collagen binding integrins activates integrin-mediated signaling via SRC kinases to induce expression of *EGR1*, resulting in transcriptional activation of the *MT1-MMP* promoter and subsequent MT1-MMP–catalyzed collagen invasion.[2] Laminin, fibronectin, and collagen can all enhance chemotactic activity associated with activation of Ras/MAP, whereas enhanced invasion is observed only with laminin and collagen. The α_3, α_6, and β_1 integrin-mediated signaling through Ras/MAP, Erk, and AKT have been implicated in chemotaxis and invasion.

Stress hormones—including epinephrine, norepinephrine, and cortisol—have been shown to upregulate MMP-2 and MMP-9 in ovarian cancer cells, enhancing invasion and angiogenesis. Chronic behavioral stress produced higher levels of tissue catecholamines, greater tumor burden, and more invasive growth in an orthotopic murine model of ovarian cancer.[51] β2 adrenergic receptor-driven cyclic AMP (cAMP)-protein kinase A (PKA) and Src signaling increase migration, invasion, and vascularization, enhancing tumor growth and increasing expression of VEGF, MMP-2, and MMP-9.[52] Moreover, among ovarian cancer patients, the use of beta blockers was significantly associated with reduced cancer-related mortality.

Ovarian cancer metastases grow efficiently within the peritoneal cavity, but not as well at other sites.[43] In the past, peritoneovenous shunts were used to palliate intractable ascites, resulting in the systemic infusion of large numbers of ovarian cancer cells. At postmortem, the majority of ovarian cancer patients did not develop widely disseminated macroscopic hematogenous metastases, consistent with the possibility that the "soil" is indeed as important as the "seed."[53] Predilection for the omentum may be explained by the ability of adipocytes to secrete adipokines including IL-8 that promote chemotaxis and invasion of ovarian cancer cells, as well as their ability to provide fatty acids as an energy source for rapid cancer growth.[54]

Angiogenesis

Angiogenesis is an important component of metastatic potential. In primary ovarian cancers, microvessel density has correlated directly with the propensity to metastasize and inversely with disease-free survival.[2] VEGF, PDGF, acidic FGF, basic FGF, angiopoietin 1 and 2, IL-6, and IL-8 can all contribute to angiogenesis in different ovarian cancers.[2,55-57] Most ovarian cancers express VEGF, which stimulates proliferation of endothelial cells and serves as a survival factor both for endothelial cells and for ovarian cancer cells that express VEGFR family members. Treatment with the anti-VEGF antibody bevacizumab has produced an objective response rate of 16% in patients with recurrent ovarian cancer and has stabilized disease for 5.5 months in 50%.[56] In randomized trials in first line, the addition of bevacizumab to conventional chemotherapy has increased progression-free survival by 2.7 to 3.8 months ($P = .004$, $P < .001$).[58,59] Robust predictive biomarkers for response to bevacizumab are still being sought. Platelet-derived growth factor (PDGF) has been detected in areas of increased blood flow in ovarian cancer.[2] Pericytes—cells that cover endothelial cells and stabilize vessels—express PDGFR and secrete VEGF, creating a paracrine loop with vascular endothelial cells that secrete PDGF and express VEGFRs. Consequently, both receptors and both cell types might be targeted for more effective antivascular therapy.

Other targets may prove useful for antivascular therapy.[57] FAK is overexpressed in more than two thirds of human ovarian cancers associated with shorter survival. In addition to enhancing migration, invasion, and metastasis of cancer cells, FAK activation increases VEGF transcription, angiogenic cytokine production, and pericyte migration. Chemical inhibition or siRNA knockdown of FAK in xenograft models has slowed cancer growth, inhibited angiogenesis, and enhanced taxane sensitivity. Dll4, one of the Delta-like ligands for NOTCH, is overexpressed in 72% of ovarian cancers and is an independent predictor of poor survival. Inhibiting Dll4 inhibits tumor growth by inducing nonproductive angiogenesis with increased vascular density and decreased perfusion of tumors. Combining Dll4-targeted siRNA with bevacizumab resulted in greater inhibition of

tumor growth, compared with bevacizumab alone in animal models. The availability of gamma secretase inhibitors and anti-Dll4 antibodies should facilitate translation to the clinic. Zeste homolog 2 (EZH2) is a polycomb protein that has been detected in tumor-associated endothelial cells in a fraction of ovarian cancers with a poor prognosis. VEGF from cancer cells induces EZH2, which methylates and silences vasohibin1 (VASH1), a potent anti-angiogenic factor.[60] Silencing EZH2 in tumor-associated endothelial cells with siRNA inhibited angiogenesis and reduced xenograft growth by reactivating VASH1. Finally, EphA2 is a transmembrane tyrosine kinase receptor that is overexpressed in 76% of ovarian cancers with later stage and higher grade. EphA2 receptor activation is required for VEGF-mediated endothelial cell migration and has been associated with vasculogenic mimicry by cancer cells. Microvessel density is increased and MMp-2 and MMP-9 increased in clinical samples that overexpress EphA2. Agonistic monoclonal antibodies, receptor-TRAPs, and siRNA have all proven effective in preclinical models.

Immunologic and Inflammatory Factors

Cytokines and Chemokines

Ovarian cancers can express up to 1000 times more TNF-α than normal ovarian epithelial cells. Some 80% of ovarian cancers express TNF-α, regulated translationally and transcriptionally through NF-κB. Ovarian cancer cells can also express both TNFRI and TNFRII, receptors that permit both autocrine and paracrine stimulation. Exogenous TNF-α or IL-1α enhances the expression of endogenous TNF-α and increases levels of IL-1α, IL-6, CCL2, CXCL8, and M-CSF.[61] TNF-α can exert contrasting effects on different ovarian cancers by inhibiting, failing to effect, or stimulating tumor cell proliferation. In 10% to 25% of tumor cells taken directly from patients, TNF-α can stimulate clonogenic growth. Knockdown of endogenous TNF-α has inhibited the growth and dissemination of ovarian cancer xenografts.[2] Clinical trials have been undertaken with infliximab, which blocks TNF-α in ovarian cancer patients. Interaction of TNF, CXCL12, and IL6 in an autocrine cytokine network can influence angiogenesis, myeloid cell infiltration, and NOTCH signaling.

Interleukin-6 (IL-6) also plays an important role in mediating paraneoplastic thrombocytosis. Thrombocytosis is associated with a poor prognosis and elevated plasma levels of thrombopoietin and IL-6.[62] Silencing thrombopoietin and interleukin-6 abrogated thrombocytosis in tumor-bearing mice. Anti–IL-6 antibody treatment significantly reduced platelet counts in tumor-bearing mice and in patients with epithelial ovarian cancer. In addition, neutralizing IL-6 significantly enhanced the therapeutic efficacy of paclitaxel in mouse models of epithelial ovarian cancer. Treatment with an anti-platelet antibody significantly reduced tumor growth and angiogenesis in tumor-bearing mice.

Immunosuppression

Immunodeficiency has been documented in patients with ovarian cancer. Advanced disease has been associated with defects in delayed cutaneous hypersensitivity and in the humoral immune response. Before treatment, T-cell numbers and subsets in peripheral blood have been comparable to controls, but functional defects of B cells have been detected.[2] Following primary chemotherapy, T-cell function is also compromised. T cells isolated from ascites fluid or tumor tissue exhibit decreased expression of the TCR-zeta chain, and downregulation of the TCR-zeta chain can be produced ex vivo by co-culture of T cells with macrophages or soluble tumor-derived factors.[2] The presence of $CD4^+CD25^+FOXP3^+$ regulatory T cells suppresses specific T-cell–mediated immunity in tumor masses but not in stroma and has been associated with decreased survival.[2] Plasmacytoid dendritic cells favor the induction of tolerance.[63] Tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates trafficking of Treg cells. Ovarian cancers can also produce TGF-β and several immunosuppressive factors including IL-10, VEGF, fibronectin, and mucins.[2]

Humoral Immune Response

Despite an immunosuppressive environment, antibodies against tumor-associated antigens can be found in the blood of ovarian cancer patients. Correlation of antibodies against MUC1 with favorable risk factors has raised the interesting hypothesis that immunity to mucins might suppress the development of ovarian cancers, although there may be many different reasons for the development of anti-MUC1 antibodies.[2] Antibodies against mesothelin, *TP53*, and HER2 have been found in ovarian cancer patients and are being explored as potential biomarkers for early diagnosis and monitoring.

Cellular Immune Response

T cells from ovarian cancer patients can kill autologous tumor cells following in vitro activation. Cytotoxic T cells can be generated by incubating peripheral blood lymphocytes with dendritic cells that have been pulsed with extracts

of autologous, but not allogeneic, ovarian cancers.[2] Cytotoxic T cells bear Fas ligand and induce apoptosis in cells that express Fas, which includes most ovarian cancers. Restricted expression of T-cell receptor V-β subtypes has been observed in tumor-associated lymphocytes, consistent with antigen-driven expansion of specific clones. Aberrantly glycosylated mucins, including MUC1, are expressed by most ovarian cancers, and T cells reactive with MUC1 and MUC16 molecules have been obtained from ovarian cancer patients. *TP*53 is mutated in approximately 70% of all ovarian cancers and in virtually all high-grade serous ovarian cancers, and T cells reactive with TP53 can be detected in some 50%, but are also found in a similar fraction of controls with benign disease.[2] T cells reactive with HER-2 epitopes have been isolated from the ascites fluid of ovarian cancer patients. Other antigens have been recognized by T cells from ovarian cancer patients, including folate receptor-α, NY-ESO-1, MAGE, Sp17, survivin, and telomerase.

T-cell antigen epitopes are recognized in the context of specific major histocompatibility complex (MHC) determinants. Normal ovarian surface epithelial cells express class I but not class II MHC components. In ovarian cancers, class I determinants are expressed in approximately 80%, and class II determinants are expressed in 40%.[2] The level of class I MHC expression in epithelial ovarian cancer cells has correlated with the degree of T-cell infiltration in vivo and the ability to expand T cells in vitro in the presence of low levels of IL-2. Low class I MHC expression is a poor prognostic factor in aneuploid ovarian cancers. Antibodies reactive with autologous tumor cells have also been identified.

Ascites contains widely varying fractions of lymphocytes, macrophages, mesothelial cells, and cancer cells. In one study, an average of 51% CD8 T cells, 10% CD4 T cells, and 27% CD14 macrophages were encountered.[2] A variety of chemokines—CCL-2, 3, 4, 5, 8, and 22—and their receptors—CCR1, 2a, 2b, 3, 4, 5, and 8—were detected, and a direct correlation was found between CCL5 and the CD3 T-cell infiltration. Chemokine CXCL12 and its unique receptor CXCR4 have been implicated in metastasis of several different cancers. CXCL12 was found in 91% of ovarian cancers and CXCR4 in 59%.[2] Expression of CXCR4 was associated with decreased disease-free and overall survival. CXCL12 and VEGF are both induced in ovarian cancer cells by hypoxia and synergize to induce tumor vessels.[2] CXCL12 also attracts plasmacytoid dendritic cells into ascites that further enhance angiogenesis by secreting IL-8 and TNF-α.[2]

Among the cells that infiltrate solid ovarian cancers, T cells are most prevalent, B cells are rare, and macrophages vary in number. In addition to the specific cytotoxic effects of T cells, the interferons produced by activated T cells can inhibit tumor growth, inhibit IL-8 secretion, block angiogenesis, upregulate MHC, and augment mucin expression. Intratumoral T cells have been found in 55% of ovarian cancers and are associated with a 5-year survival rate of 38%, compared with 4.5% for patients whose tumors lack T-cell infiltrates.[2] Both CD4 and CD8 cells can be found at tumor sites. The presence of CD8 T cells and a high CD8/CD4 ratio has correlated with the most favorable prognosis, related to the adverse effect of $CD4^+CD25^+FOXP3^+$ regulatory T cells within the $CD4^+$ population.[2] Ovarian cancer cells can secrete M-CSF and MCP1 that exert potent chemotactic activity for macrophages. Cytokines and factors released from activated macrophages can stimulate (IL-1, IL-6, TNF) or inhibit (nitric oxide, TNF) tumor growth. Tumor-associated macrophages have impaired phagocytic activity and effector function for ADCC. Cytotoxic NK cells have been detected in ascites fluid and in solid ovarian cancers. Despite these many potential immune effector mechanisms, most ovarian cancers grow progressively.

Based on the immunobiology of ovarian cancer, a number of strategies have been evaluated for the treatment of the disease.[64] Cancer vaccines have included idiotype anti-MUC16 (CA125),[65] MUC1, CEA, and NY-ESO-1. An alternative approach has used autologous ovarian cancer extracts, viral oncolysates or primed dendritic cells with or without depletion of T_{REGS} using metronomic daily cyclophosphamide. Adoptive immunotherapy has been performed using tumor infiltrating lymphocytes (TILs) expanded ex vivo with IL-2. In an early nonrandomized trial, consolidation of primary therapy with TILs and IL-2 improved progression-free and overall survival in a small group of ovarian cancer patients. Substantial effort has been directed in the laboratory toward developing methods for optimal expansion and antigen-specific stimulation of TILs, as well as to introducing T-cell receptors or chimeric antigen receptors (CARs) into T cells. Monoclonal antibodies have been used to regulate T-cell activity. Ipilimumab, an anti-CTLA4 that inactivates a T-cell checkpoint, has produced anecdotal responses in ovarian cancer patients.

Conclusion

Despite major progress over the past decade, many critical questions remain to be answered if we are to develop therapeutic approaches that will optimally benefit patients. An understanding of the pathogenesis of epithelial ovarian cancer should permit earlier detection and more effective, potentially less toxic, therapy. We will need to embrace the concept that ovarian cancer consists of multiple independent diseases with two major subgroups of Type I and Type II, with the corollary that clinical trials and translational studies must be

performed independently on each disease type. Biomarkers in addition to CA125 and imaging techniques with higher resolution than TVS must be identified to improve the ability to detect small volumes of disease in the ovary or fallopian tube for both early diagnosis and patient monitoring. Autoantibodies against overexpressed wild-type proteins and mutant TP53 provide attractive biomarker candidates, and SQUID imaging with targeted magnetic nanoparticles poses a potentially transformative technology. Low-grade Type I cancers are driven by Ras mutations, PI3K activation, and paracrine IGF signaling, in the context of wild-type TP53 and expression of ER and PR. Combinations of drugs that inhibit MEK, the PI3K pathway, and IGFR need to be tested in Type I ovarian cancers. Hormonal therapy, particularly in the context of targeted therapy, should be explored, especially considering the recent outcomes of trials of hormonal manipulation and mTOR targeting in breast cancers. High-grade Type II ovarian cancers are driven by copy number abnormalities that affect PI3K, NOTCH, and other pathways with loss of TP53 and/or BRCA1/2 function. Dual inhibition of PI3Kness and BRCAness should be evaluated in Type II ovarian cancers based on synergistic activity seen animal models. A variety of drugs targeting the PI3K pathway are in trials in ovarian cancer, and PAPR inhibitors that act as synthetic lethal with defects in BRCA1/2 or homologous recombination have shown activity in selected patients—providing an opportunity for combination trials, which indeed have just been initiated. Development of strategies that target mutant TP53 or act as a "synthetic lethal" with mutant TP53 constitutes a significant knowledge gap that needs to be filled. A number of compounds that can bind and potentially normalize function of TP53 with specific hotspot mutations have been developed and are entering clinical trials. Relevant genetically engineered murine models are needed that are driven by copy number abnormalities. Robust biomarkers for stem cell–like cells must be identified and strategies devised to eliminate dormant cancer cells. Predictive biomarkers are also needed to identify patients most likely to benefit from bevacizumab. Several other angiogenic targets must be evaluated, including FAK, Dll4, EZH2, and EphA2. Finally, therapy with vaccines, adoptive therapy, and checkpoint and other immunoregulatory antibodies must be combined strategically with targeted agents.

Acknowledgments

These studies were supported in part by a grant from the National Cancer Institute R01 CA135354, by the M.D. Anderson SPORE in Ovarian Cancer NCI P50 CA83639, the Shared Resources of the M.D. Anderson CCSG NCI P30 CA16672, the Ovarian Cancer Research Fund, the National Foundation for Cancer Research, philanthropic support from the Zarrow Foundation and Stuart and Gaye Lynn Zarrow, Golfers Against Cancer, the Kaye Yow Foundation, and the Mossy Family Foundation.

Key References

1. Siegel R, Naishadam D, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2013;63:11-30.
2. Bast Jr RC, Mills GB. Molecular pathogenesis of epithelial ovarian cancer. In: Mendelsohn J, Howley P, Israel M, Gray JW, Thompson CB, eds. *The Molecular Basis of Cancer*. 3rd ed. Philadelphia, Pa: Saunders-Elsevier; 2008:441-454.
3. Kurman RJ. Shih IeM. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer—shifting the paradigm. *Hum Pathol*. 2011;42:918-931.
4. Crum CP, Drapkin R, Kindelberger D, et al. Lessons from BRCA: the tubal fimbria emerges as the origin of pelvic serous cancer. *Clin Med Res*. 2007;5:35-44.
10. See HT, Kavanagh JJ, Hu W, et al. Targeted therapy for epithelial ovarian cancer: current status and future prospects. *Int J Gynecol Cancer*. 2003;13:701-734.
11. Marquez RT, Baggerly KA, Patterson AP, et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium and colon. *Clin Cancer Res*. 2005;11:6116-6126.
12. Cheng W, Liu J, Yoshida H, et al. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med*. 2005;11:531-537.
13. Landen Jr CN, Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. *J Clin Oncol*. 2008;26:995-1005.
14. Bast Jr RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer*. 2009;9:415-428.
15. Romero IN, Bast Jr RC. Minireview: Human ovarian cancer: biology, current management and paths to personalized therapy. *Endocrinology*. 2012;153:1593-1602.
16. Vaughan S, Coward JI, Bast Jr RC, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer*. 2011;11:719-725.
17. Gershenson DM, Sun CC, Bodurka DM, et al. Recurrent low-grade serous ovarian carcinoma is relatively chemoresistant. *Gynecol Oncol*. 2009;114:48-52.
18. Zorn KK, Bonome T, Gangi L, et al. Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer. *Clin Cancer Res*. 2005;11:6422-6430.
19. Jones S, Wang TL, IeM Shih, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330:228-231.
20. Wiegand KC, Shah SP, Al-Agha OM, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med*. 2010;363:1532-1543.
21. Shah SP, Kobel M, Senz J, et al. Mutation of FOXL2 in granulosa cell tumors of the ovary. *N Engl J Med*. 2009;360:2719-2729.
22. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609-615.
23. Bast RC, Mills GB. Dissecting PI3Kness: the complexity of personalized therapy for ovarian cancer. *Cancer Discov*. 2012;2:16-18.
24. Bast RC, Mills GB. Personalizing therapy for ovarian cancer: BRCAness and beyond. *J Clin Oncol*. 2010;28:3545-3548.
25. Tothill RW, Tinker AV, George J, et al. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res*. 2008;14:5198-5208.
26. Verhaak RG, Tamayo P, Yang JY, et al. Prognostically relevant gene signatures of high grade serous ovarian cancer. *J Clin Invest*. 2013;123:517-525.

27. Yang G, Rosen DG, Colacino JA, et al. Disruption of the retinoblastoma pathway by small interfering RNA and ectopic expression of the catalytic subunit of telomerase lead to immortalization of human ovarian surface epithelial cells. *Oncogene*. 2007;26:1492-1498.
28. Merritt WM, Lin YG, Han LY, et al. Dicer, drosha and outcomes in patients with ovarian cancer. *N Engl J Med*. 2008;359:2641.
29. Heravi-Moussavi A, Anglesio MS, Cheng SW, et al. Recurrent somatic *DICER1* mutations in nonepithelial ovarian cancers. *N Engl J Med*. 2012;366:234-242.
30. Balch C, Fang F, Matei DE, et al. Minireview: Epigenetic changes in ovarian cancer. *Endocrinology*. 2009;150:4003-4011.
32. Yu Y, Luo R, Lu Z, et al. Biochemistry and biology of ARHI (DIRAS3), an imprinted tumor suppressor gene whose expression is lost in ovarian and breast cancers. In: Balch WE, Der C, Hall A, eds. *Methods in Enzymology, Regulators and Effectors of Small GTPases. Part D. Ras Proteins*. New York, NY: Academic Press; 2006:455.
33. Lu Z, Luo RZ, Lu Y, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest*. 2008;118:3917-3929.
34. Ahmed AA, Lu Z, Jennings NB, et al. SIK2 is a centrosome kinase required for bipolar mitotic spindle formation that provides a potential target for therapy in ovarian cancer. *Cancer Cell*. 2010;18:109-121.
35. Zhang X, George J, Deb S, et al. The Hippo pathway transcriptional co-activator YAP is an ovarian cancer oncogene. *Oncogene*. 2011;30:2810-2822.
36. Zaid TM, Yeung T-L, Thompson MS, et al. Identification of FGFR4 as a potential therapeutic target for advanced-stage high grade serous ovarian carcinoma. *Clin Cancer Res*. 2013;19:809-820.
37. Pua TL, Wang FQ, Fishman DA. Roles of LPA in ovarian cancer development and progression. *Future Oncol*. 2009;5:1659-1673.
38. Burgos-Ojeda D, Rueda BR, Buckanovich RJ. Ovarian cancer stem cell markers: prognostic and therapeutic stem cell markers. *Cancer Lett*. 2012;322:1-7.
39. Flesken-Nikiten A, Hwang C-I, Cheng C-Y, et al. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature*. 2013;495:241-245.
40. Mullany LK, Richards JS. Minireview: Animal models and mechanisms of ovarian cancer development. *Endocrinology*. 2012;153:1585-1592.
41. El Masri WM, Csagrande G, Hoskins E, et al. Cell adhesion in ovarian cancer. *Cancer Treat Res*. 2009;149:297-318.
42. Barbolina MV, Moss NM, Westfall SD, et al. Microenvironmental regulation of ovarian cancer metastases. *Cancer Treat Res*. 2009;149:319-334.
43. Lengyel E. Ovarian cancer development and metastasis. *Am J Pathol*. 2010;177:1059-1064.
44. Bast RC, Spriggs DR. More than a biomarker: CA125 may contribute to ovarian cancer pathogenesis. *Gynecol Oncol*. 2011;121:429-430.
46. Bast Jr RC. Commentary: CA125 and the detection of recurrent ovarian cancer: a reasonably accurate biomarker for a difficult disease. *Cancer*. 2010;116:2850-2853.
47. Buys SS, Partridge E, Black A, et al. Effect of screening on ovarian cancer mortality: the prostate, lung, colon and ovarian (PLCO) cancer screening randomized trial. *JAMA*. 2011;302:2295-2303.
48. Menon U, Gentry Maharaj A, Hallett R, et al. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). *Lancet Oncol*. 2009;10:327-340.
49. Yurkovetsky Z, Skates S, Lomakin A, et al. Development of a multimarker assay for detection of ovarian cancer. *J Clin Oncol*. 2010;28:2159-2166.
50. Mitra AK, Sawada K, Tiwari P, et al. Ligand independent activation of c-Met by fibronectin and α5β1 integrin regulates ovarian cancer invasion and metastasis. *Oncogene*. 2010;30:1566-1576.
51. Thaker PH, Han LY, Kamal AA, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian cancer. *Nat Med*. 2006;12:939-944.
52. Amaiz-Pena GN, Allen JK, Cruz A, et al. Src activation by β-adrenoreceptors is a key switch for tumour metastasis. *Nat Commun*. 2013;4:1403.
54. Nieman KM, Kenny HA, Penicka CV, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumour growth. *Nat Med*. 2011;17:1498-1503.
56. Schmitt J, Matei D. Targeting angiogenesis in ovarian cancer. *Cancer Treat Rev*. 2012;38:272-283.
57. Thanapprapasr D, Hu W, Sood AK, et al. Moving beyond VEGF for anti-angiogenesis strategies in gynecologic cancer. *Current Pharm Des*. 2012;18:2713-2719.
58. Perren TJ, Swart AM, Pfisterer J, et al. A phase 3 trial of bevacizumab in ovarian cancer. *N Engl J Med*. 2011;365:2484-2496.
59. Burger RA, Brady MF, Bookman MA, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N Engl J Med*. 2011;365:2473-2483.
60. Lu C, Han HD, Mangala LS, et al. Regulation of tumor angiogenesis by EZH2. *Cancer Cell*. 2010;18:185-197.
61. Kulbe H, Thompson R, Wilson JL, et al. The inflammatory cytokine tumor necrosis factor-alpha generates and autocrine tumor-promoting network in epithelial ovarian cancer cells. *Cancer Res*. 2007;67:585-592.
62. Stone RL, Nick AM, McNeish IA, et al. Paraneoplastic thrombocytosis in ovarian cancer. *N Engl J Med*. 2012;366:610-618.
63. Yigit R, Massegur LFAG, Figdor CG, et al. Ovarian cancer creates a suppressive microenvironment to escape immune elimination. *Gynecol Oncol*. 2010;117:366-372.
64. Kandalaft LE, Powell Jr DJ, Singh N, et al. Immunotherapy of ovarian cancer: What's next? *J Clin Oncol*. 2011;29:925-933.
65. Grisham RN, Berek J, Pfisterer J, et al. Abagovomab: an anti-idiotypic CA-125 targeted therapeutic agent for ovarian cancer. *Immunotherapy*. 2011;3:153-162.

This page intentionally left blank

References

1. Siegel R, Naishadam D, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2013;63:11-30.
2. Bast Jr RC, Mills GB. Molecular pathogenesis of epithelial ovarian cancer. In: Mendelsohn J, Howley P, Israel M, Gray JW, Thompson CB, eds. *The Molecular Basis of Cancer*. 3rd ed. Philadelphia, Pa: Saunders-Elsevier; 2008:441-454.
3. Kurman RJ. Shih IeM. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer—shifting the paradigm. *Hum Pathol*. 2011;42:918-931.
4. Crum CP, Drapkin R, Kindelberger D, et al. Lessons from BRCA: the tubal fimbria emerges as the origin of pelvic serous cancer. *Clin Med Res*. 2007;5:35-44.
5. Kohler MF, Marks JR, Wiseman RW, et al. Spectrum of mutation and frequency of allelic deletion of the p53 gene in ovarian cancer. *J Natl Cancer Inst*. 1993;85:1513-1519.
6. Knapp RC, et al. Natural history and detection of ovarian cancer. In: Sciarra JW, ed. *Gynecology and Obstetrics, Vol iv: Oncology*. Philadelphia, PA: Harper & Row; 1988:2.
7. Mesiano S, Ferrara N, Jaffe R. Role of vascular endothelial growth factor in ovarian cancer: Inhibition of ascites formation by immunoneutralization. *Am J Pathol*. 1998;153:1249-1256.
8. Goff BA, Mandel L, Muntz HG, et al. Ovarian carcinoma diagnosis. *Cancer*. 2000;89:2068-2075.
9. Jacobs IJ, Kohler MF, Wiseman RW, et al. Clonal origin of epithelial ovarian carcinoma: analysis by loss of heterozygosity, p53 mutation, and X-chromosome inactivation. *J Natl Cancer Inst*. 1984:1793-1798.
10. See HT, Kavanagh JJ, Hu W, et al. Targeted therapy for epithelial ovarian cancer: current status and future prospects. *Int J Gynecol Cancer*. 2003;13:701-734.
11. Marquez RT, Baggerly KA, Patterson AP, et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium and colon. *Clin Cancer Res*. 2005;11:6116-6126.
12. Cheng W, Liu J, Yoshida H, et al. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med*. 2005;11:531-537.
13. Landen Jr CN, Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. *J Clin Oncol*. 2008;26:995-1005.
14. Bast Jr RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer*. 2009;9:415-428.
15. Romero IN, Bast Jr RC. Minireview: Human ovarian cancer: biology, current management and paths to personalized therapy. *Endocrinology*. 2012;153:1593-1602.
16. Vaughan S, Coward JI, Bast Jr RC, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer*. 2011;11:719-725.
17. Gershenson DM, Sun CC, Bodurka DM, et al. Recurrent low-grade serous ovarian carcinoma is relatively chemoresistant. *Gynecol Oncol*. 2009;114:48-52.
18. Zorn KK, Bonome T, Gangi L, et al. Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer. *Clin Cancer Res*. 2005;11:6422-6430.
19. Jones S, Wang TL, IeM Shih, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. 2010;330:228-231.
20. Wiegand KC, Shah SP, Al-Agha OM, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med*. 2010;363:1532-1543.
21. Shah SP, Kobel M, Senz J, et al. Mutation of FOXL2 in granulosa cell tumors of the ovary. *N Engl J Med*. 2009;360:2719-2729.
22. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609-615.
23. Bast RC, Mills GB. Dissecting PI3Kness: the complexity of personalized therapy for ovarian cancer. *Cancer Discov*. 2012;2:16-18.
24. Bast RC, Mills GB. Personalizing therapy for ovarian cancer: BRCAness and beyond. *J Clin Oncol*. 2010;28:3545-3548.
25. Tothill RW, Tinker AV, George J, et al. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res*. 2008;14:5198-5208.
26. Verhaak RG, Tamayo P, Yang JY, et al. Prognostically relevant gene signatures of high grade serous ovarian cancer. *J Clin Invest*. 2013;123:517-525.
27. Yang G, Rosen DG, Colacino JA, et al. Disruption of the retinoblastoma pathway by small interfering RNA and ectopic expression of the catalytic subunit of telomerase lead to immortalization of human ovarian surface epithelial cells. *Oncogene*. 2007;26:1492-1498.
28. Merritt WM, Lin YG, Han LY, et al. Dicer, drosha and outcomes in patients with ovarian cancer. *N Engl J Med*. 2008;359:2641.
29. Heravi-Moussavi A, Anglesio MS, Cheng SW, et al. Recurrent somatic *DICER1* mutations in nonepithelial ovarian cancers. *N Engl J Med*. 2012;366:234-242.
30. Balch C, Fang F, Matei DE, et al. Minireview: Epigenetic changes in ovarian cancer. *Endocrinology*. 2009;150:4003-4011.
31. Yu Y, Xu F, Peng H, et al. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci USA*. 1999;96:214-219.
32. Yu Y, Luo R, Lu Z, et al. Biochemistry and biology of ARHI (DIRAS3), an imprinted tumor suppressor gene whose expression is lost in ovarian and breast cancers. In: Balch WE, Der C, Hall A, eds. *Methods in Enzymology, Regulators and Effectors of Small GTPases. Part D. Ras Proteins*. New York, NY: Academic Press; 2006:455.
33. Lu Z, Luo RZ, Lu Y, et al. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *J Clin Invest*. 2008;118:3917-3929.
34. Ahmed AA, Lu Z, Jennings NB, et al. SIK2 is a centrosome kinase required for bipolar mitotic spindle formation that provides a potential target for therapy in ovarian cancer. *Cancer Cell*. 2010;18:109-121.
35. Zhang X, George J, Deb S, et al. The Hippo pathway transcriptional co-activator YAP is an ovarian cancer oncogene. *Oncogene*. 2011;30:2810-2822.
36. Zaid TM, Yeung T-L, Thompson MS, et al. Identification of FGFR4 as a potential therapeutic target for advanced-stage high grade serous ovarian carcinoma. *Clin Cancer Res*. 2013;19:809-820.
37. Pua TL, Wang FQ, Fishman DA. Roles of LPA in ovarian cancer development and progression. *Future Oncol*. 2009;5:1659-1673.
38. Burgos-Ojeda D, Rueda BR, Buckanovich RJ. Ovarian cancer stem cell markers: prognostic and therapeutic stem cell markers. *Cancer Lett*. 2012;322:1-7.
39. Flesken-Nikiten A, Hwang C-I, Cheng C-Y, et al. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature*. 2013;495:241-245.
40. Mullany LK, Richards JS. Minireview: Animal models and mechanisms of ovarian cancer development. *Endocrinology*. 2012;153:1585-1592.
41. El Masri WM, Csagrande G, Hoskins E, et al. Cell adhesion in ovarian cancer. *Cancer Treat Res*. 2009;149:297-318.
42. Barbolina MV, Moss NM, Westfall SD, et al. Microenvironmental regulation of ovarian cancer metastases. *Cancer Treat Res*. 2009;149:319-334.
43. Lengyel E. Ovarian cancer development and metastasis. *Am J Pathol*. 2010;177:1059-1064.
44. Bast RC, Spriggs DR. More than a biomarker: CA125 may contribute to ovarian cancer pathogenesis. *Gynecol Oncol*. 2011;121:429-430.
45. Bast Jr RC, Klug TL, St. John E, et al. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *N Engl J Med*. 1983;309:883-887.
46. Bast Jr RC. Commentary: CA125 and the detection of recurrent ovarian cancer: a reasonably accurate biomarker for a difficult disease. *Cancer*. 2010;116:2850-2853.
47. Buys SS, Partridge E, Black A, et al. Effect of screening on ovarian cancer mortality: the prostate, lung, colon and ovarian (PLCO) cancer screening randomized trial. *JAMA*. 2011;302:2295-2303.
48. Menon U, Gentry Maharaj A, Hallett R, et al. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). *Lancet Oncol*. 2009;10:327-340.
49. Yurkovetsky Z, Skates S, Lomakin A, et al. Development of a multimarker assay for detection of ovarian cancer. *J Clin Oncol*. 2010;28:2159-2166.
50. Mitra AK, Sawada K, Tiwari P, et al. Ligand independent activation of c-Met by fibronectin and α5β1 integrin regulates ovarian cancer invasion and metastasis. *Oncogene*. 2010;30:1566-1576.
51. Thaker PH, Han LY, Kamal AA, et al. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian cancer. *Nat Med*. 2006;12:939-944.
52. Amaiz-Pena GN, Allen JK, Cruz A, et al. Src activation by β-adrenoreceptors is a key switch for tumour metastasis. *Nat Commun*. 2013;4:1403.

53. Tarin D, Price JE, Kettlewell MG, et al. Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. *Cancer Res*. 1984; 44:3584.
54. Nieman KM, Kenny HA, Penicka CV, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumour growth. *Nat Med*. 2011;17:1498-1503.
55. Yoneda J, Kuniyasu H, Crispens M, et al. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst*. 1998;90:447-454.
56. Schmitt J, Matei D. Targeting angiogenesis in ovarian cancer. *Cancer Treat Rev*. 2012;38:272-283.
57. Thanapprapasr D, Hu W, Sood AK, et al. Moving beyond VEGF for anti-angiogenesis strategies in gynecologic cancer. *Current Pharm Des*. 2012;18:2713-2719.
58. Perren TJ, Swart AM, Pfisterer J, et al. A phase 3 trial of bevacizumab in ovarian cancer. *N Engl J Med*. 2011;365:2484-2496.
59. Burger RA, Brady MF, Bookman MA, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N Engl J Med*. 2011;365:2473-2483.
60. Lu C, Han HD, Mangala LS, et al. Regulation of tumor angiogenesis by EZH2. *Cancer Cell*. 2010;18:185-197.
61. Kulbe H, Thompson R, Wilson JL, et al. The inflammatory cytokine tumor necrosis factor-alpha generates and autocrine tumor-promoting network in epithelial ovarian cancer cells. *Cancer Res*. 2007;67:585-592.
62. Stone RL, Nick AM, McNeish IA, et al. Paraneoplastic thrombocytosis in ovarian cancer. *N Engl J Med*. 2012;366:610-618.
63. Yigit R, Massegur LFAG, Figdor CG, et al. Ovarian cancer creates a suppressive microenvironment to escape immune elimination. *Gynecol Oncol*. 2010;117:366-372.
64. Kandalaft LE, Powell Jr DJ, Singh N, et al. Immunotherapy of ovarian cancer: What's next? *J Clin Oncol*. 2011;29:925-933.
65. Grisham RN, Berek J, Pfisterer J, et al. Abagovomab: an anti-idiotypic CA-125 targeted therapeutic agent for ovarian cancer. *Immunotherapy*. 2011;3:153-162.

Long-Cheng Li, Andrew C. Hsieh, Davide Ruggero, Kirsten L. Greene, and Peter R. Carroll

38

Molecular Basis of Prostate Cancer

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer death in men in the United States.[1] Prostate-specific antigen (PSA) screening began in the late 1980s and dramatically increased the diagnosis of this disease. An almost-simultaneous decrease in disease-specific mortality has been noted.[2] Whether this is a result of early and enhanced screening, early treatment of localized disease, early and aggressive treatment of micrometastatic disease, or other unknown reasons is a matter of considerable debate. Screening remains widespread but controversial because of conflicting evidence demonstrating overall survival benefit.[3,4] Prostate cancer has a very heterogeneous natural history, and screening has resulted in overdetection and overtreatment of men with indolent prostate cancer.[3,4] Prostate cancer is not, as yet, curable once it has metastasized; however, differentiation of early-stage disease that ultimately will progress from disease destined to remain indolent is a major research priority. The molecular genetics of prostate cancer hold promise for the development of new screening and diagnostic tests to resolve this issue.

Several risk factors have been associated with prostate cancer, including age, race, family history, and obesity. Subsequently, tumor suppressors, oncogenes, and polymorphisms have been analyzed to help explain these risk factors. Epigenetic alterations have been observed in prostate cancer affecting the expression and function of a large array of genes involved in tumorigenesis, tumor progression, and metastasis. The search for new and more specific biomarkers of disease continues with increased emphasis on epigenetic and genomic alterations predictive of metastasis and aggressiveness in this heterogeneous malignancy.

Pathology

Gleason Grade

The Gleason grading system is the most commonly used pathologic grading system for adenocarcinoma of the prostate. Tissue samples are examined under low magnification, and the two most common gland architectural patterns are assigned a grade from 1 to 5 and reported as a Gleason score. Figure 38-1 demonstrates the three most common Gleason scores. Gleason 1 and 2 are rarely seen in contemporary series of patients. Pathologic Gleason grade is the most important prognostic variable to the clinical risk assessment of newly diagnosed prostate cancer, followed by tumor volume. The importance of Gleason pattern 4 and 5 volume has been correlated with subsequent pathologic stage, metastasis, and outcome. Despite the decrease in prostate cancer–specific mortality seen in contemporary series, there has been a paradoxical increase in the diagnosis of patients with higher Gleason grade disease. This may be due in part to changes in pathologic criteria for diagnosing Gleason 4 disease, rather than a true increase in the incidence of higher grade disease as a result of the International Society of Uropathologists (ISUP) modifying their recommendations on histological grading of prostate cancer by expanding criteria of grade 4 disease. After this recommendation was published, it was projected that many patients previously classified as 3+3 would be subsequently upgraded to Gleason 3+4.

Prostate cancer is often multifocal, meaning that usually, multiple, distinct areas of malignancy exist within the prostate gland.[5] The largest focus of disease is often called the index tumor, and the size of this tumor has been used in disease prognostication and prediction of metastasis. An analysis of genetic alterations in tumor foci and metastases found that metastases were usually homologous with at least one tumor focus, but it was not always the index tumor. Several studies have described genetic heterogeneity within dominant tumor nodules and showed chromosomal differences between various areas of the same disease focus. Because of the genetic variability of prostate cancer and its multifocal nature, debate continues regarding the importance of smaller tumors and their impact on tumor progression and patient survival. There is genetic heterogeneity within dominant tumor nodules and chromosomal differences between various areas of the same disease focus. Because of the genetic

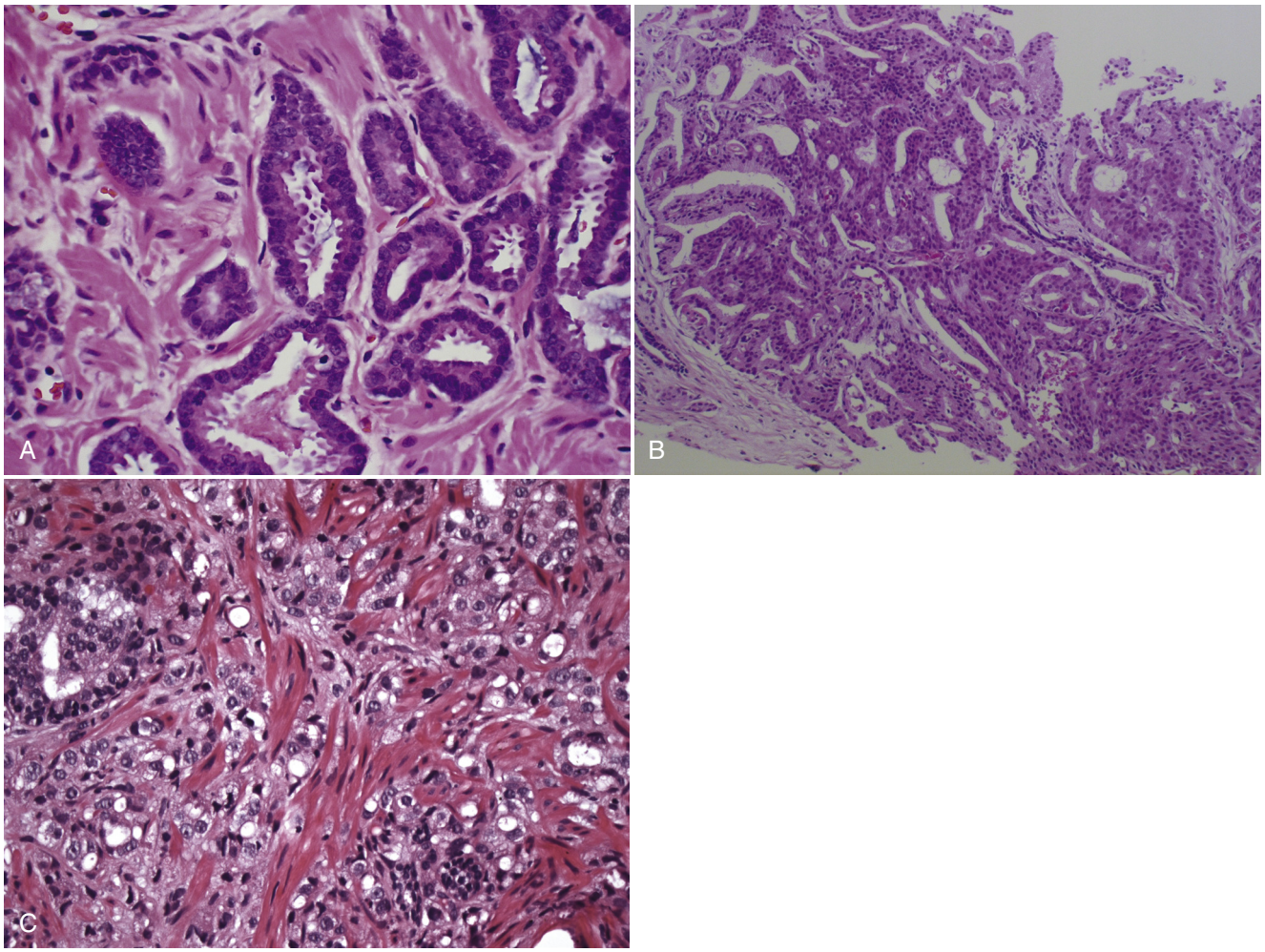


Figure 38-1 Examples of Gleason grade 3, 4, and 5 prostate cancer. Gleason grade 3 shows well-formed, separate glands **(A)**. Gleason grade 4 shows merging or cribriform glands **(B)**. Gleason grade 5 is the most poorly differentiated, and cancer cells no longer form glands but are visible as sheets of cells **(C)**.

variability of prostate cancer as well as its multifocal nature, debate continues regarding the importance of smaller tumors and their impact on tumor progression and patient survival.

Molecular Pathology

Hereditary Prostate Cancer

Family history is one of the strongest risk factors for the development of prostate cancer, with a two- to eightfold higher risk of prostate cancer in men with an affected first-degree relative.[6] Prostate cancer associated with familial clustering and high incidence of cancer among multiple first-degree relatives with a diagnosis before age 60 is considered hereditary and/or familial prostate cancer (HPC/FPC). Approximately 9% of all cases are attributable to hereditary prostate cancer following an autosomal dominant susceptibility pattern. Prostate cancer susceptibility genes have been identified using lineage analysis of affected families. Significant linkage between chromosome 1q24-25, the HPC1 locus, and hereditary prostate cancer has been established. *RNASEL*, which lies within the HPC1 locus, encodes an endoribonuclease that mediates the activities of an interferon-inducible RNA degradation pathway. Polymorphisms of the *RNASEL* gene have been associated with increased prostate cancer risk. However, not all studies have confirmed these findings. Mutations in the RNASEL gene do not occur at a greater frequency in patients with familial prostate cancer compared with patients with sporadic prostate cancer. Recent genome-wide association studies (GWAS) consistently identified that several single-nucleotide polymorphisms (SNPs) in the 8q24 locus are associated with risk of HPC/FPC22. Genetic variants caused by polymorphisms or mutations in other genes, such as *PALB2*, *BRCA2*, the androgen receptor (AR), 5-α-reductase type II (SRD5A2), and *CYP17*, have also been implicated in the development of HPC/FPC.

Table 38-1 Oncogenes and Tumor Suppressors Implicated in Prostate Cancer

Gene	Chromosome/Locus	Function	
PCA3 (DD3)	9q21–22	ncRNA with unknown function	Oncogene
EZH2	7q35	Gene silencing by histone modification	Oncogene
NKX3-1 (NKX3.1)	8p21	Homeobox gene, regulates epithelial growth and differentiation	Tumor suppressor
PTEN	10q23	Dual specificity protein/3-lipid phosphatase	Tumor suppressor
CDKN1B (p27)	12p11–13	Cyclin-dependent kinase inhibitor	Tumor suppressor
KLF6	10p15	Zinc finger transcription factor	Tumor suppressor
ERG/ETV1 (*ETS* family *TMPRSS2-ERG*)	21q22.3/7p21.2	Androgen-responsive fusion protein	Fusion oncogene
RB1 (RB)	13Q14–1-14–2	Suppress cell division	Tumor suppressor
TP53 (p53)	17p13	Cell cycle control	Tumor suppressor
CSMD1	8p23 loss	CUB and Sushi multiple domains 1	Tumor suppressor
MAP4K2	11q13.1 gain	Mitogen-activated protein kinase 2	Oncogene
MEN1	11q13.1 gain	Multiple endocrine neoplasia	Oncogene
SF1	11q13.1 gain	Splicing factor 1	Oncogene
PPP2R5B	11q13.1 gain	Protein phosphatase2, regulatory subunit B isoform	Oncogene
NAALADASEL	11q13.1 gain	*N*-acetylated α-linked acidic dipeptidase-like	Oncogene
EHD1	11q13.1 gain	EH-domain containing 1	Oncogene

Gene Mutation

Early candidate gene approaches have implicated many different genes in prostate cancer. Germline mutations involving *ELAC2* (*HPC2*), *MSR1*, and *RNASEL* genes have been reported in familial prostate cancer. The most common somatic mutations found in sporadic prostate cancer include *TP53*, *PTEN*, and *AR*. Recent whole-genome exon sequencing analyses identified significant mutated genes, including TP53, AR, ZFHX3, RB1, PTEN, APC, MLL2, OR5L1, and CDK12. Of these genes, MLL2, OR5L1, and CDK12 have unknown tumor suppressor functions in prostate cancer.[7] The most commonly affected signaling pathways by genetic alterations are the WNT signaling (TP53, APC, CTNNB1, MYC, and SMAD4) and the PTEN interaction network (PTEN, MAGI3, and HDAC11).[7]

DNA Copy Number Variation

DNA copy number variation (CNV) is DNA structure alteration involving relatively large (at least 1 kb) regions. CNV can manifest as loss or gain of chromosomal regions. Earlier studies using fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) identified common losses at 1p, 6q, 8p, 10q, 13q, 16q, and 18q and gains at 1q, 2p, 7, 8q, 18q, and Xq. For example, *MSR1*, *NKX3.1*, and *N33* are candidate tumor suppressor genes in prostate cancer lying within the most commonly deleted regions on chromosome 8p. *MSR1* encodes a receptor on the macrophage cell surface that induces binding of oxidized low-density lipoprotein and other polyanionic ligands. Mutations, polymorphisms, or loss of the *MSR1* gene may compromise global macrophage function, thereby exposing organs, including the prostate, to oxidative stress and damage. Although this gene does not code for prostatic proteins directly, oxidative stress has been implicated in the initiation of prostate carcinogenesis.

Loss of 8p23, a region that harbors the *CUB* and Sushi multiple domains 1 gene (*CSMD1*), has been associated with advanced prostate cancer. The retinoblastoma (RB1) gene is also a tumor suppressor gene and lies within the 13q locus. It is deleted in early prostate cancer development in animal models, prostate cancer cell lines, and some human prostate cancer specimens. RB1 inactivation in prostate cancer is the result of loss of heterozygosity and mutation. The 10q locus is lost in up to 45% of prostate cancers examined, and *MXI1* and *PTEN* are two putative tumor suppressors in this region.

Regions of chromosome amplification in advanced prostate cancer include 8q containing the *MYC* gene and Xq11-13 encoding the AR gene. Gene amplification at 11q13.1 has been associated with disease recurrence. There are several candidate genes in this location (Table 38-1), but only *MEN1* and *MAP4K2* correlate with disease progression.

Recent prostate cancer genome sequencing studies consistently show that the genes most commonly affected by loss of copy number are CHD1, NTSE, PTEN, RB1, and TP53, and the most gained genes are AR, MYC, PIK3CA, and the HOXA3 cluster. These aberrations are more prominent in castration-resistant prostate cancer compared to localized disease.[7,8]

Gene Fusion

The prostate cancer genome can harbor an average of 90 chromosomal rearrangements involving many genes. One of the most frequent gene fusion events in prostate cancer is the fusion of the *TMPRSS2* and *ERG* genes, which are located 3 Mb apart on chromosome 21q22.2.[9] TMPRSS2 is an androgen-responsive gene, and ERG encodes an erythroblast transformation-specific (ETS) transcription factor. Their fusion is believed to be stimulated by androgens that recruit AR and TOP2B topoisomerase to chromosomal sites where TOP2B introduces double-strand breaks in DNA. TMPRSS2-ERG fusion results in overexpression of *ETS* genes in prostate cancer. *TMPRSS2-ERG* has been identified in 40% to 70% of prostate cancer and correlated with metastasis and disease-specific mortality. Gene fusion events such as *TMPRSS2-ERG* may be used for prostate cancer diagnosis through simple PCR detection of gene fusions in urine sediment.

WNT Signaling and β-Catenin

The Wnt signaling pathway plays a key role in embryonic development and is essential for the maintenance of stem cells. Wnt is an extracellular protein that interacts with the membrane-bound frizzled receptor to initiate its biologic activity. Wnt signaling leads to stabilization of *CTNNB1* and its nuclear accumulation. Nuclear *CTNNB1* converts the TCF/LEF DNA-binding protein complex from a transcriptional repressor into a transcriptional activator. Inappropriate activation of the Wnt pathway is observed in many cancers and is putatively associated with tumor development.

In mice, *CTNNB1* stabilization through targeted excision of *CTNNB1* exon 3 induces prostate intraepithelial neoplasia (PIN)-like lesions that are similar to the early stages of human prostate cancer. In human prostate cancers, high levels of nuclear *CTNNB1* are detectable by immunohistochemistry, whereas their levels are undetectable in normal prostate tissue. High levels of *CTNNB1* expression are associated with the more aggressive prostate tumors. Together, these findings imply that inappropriate activation of the Wnt signaling pathway can contribute to prostate cancer and progression.

There are several mechanisms by which the Wnt pathway may be inappropriately activated in prostate cancer; DNA methylation plays a key role in several of these processes. The adenomatous polyposis coli (APC) gene is hypermethylated in prostate tumors relative to samples of benign prostatic hyperplasia (BPH; 64.1% vs. 8.7%). APC is a key component of the *CTNNB1* degradation complex. Thus, methylation-dependent silencing of APC can lead to *CTNNB1* accumulation and Wnt pathway activation.

E-cadherin (CDH1), a cell membrane protein, interacts with *CTNNB1* and sequesters it at the inside surface of the cellular membrane. However, CDH1 expression is often lost in prostate cancers because of chromosomal loss or promoter hypermethylation. Thus, because CDH1 is no longer present, *CTNNB1* is released into the cytoplasmic and nuclear compartments, leading to Wnt pathway activation. Finally, the secreted-frizzled related proteins (SFRPs) and Wnt inhibitory factor-1 (Wif-1) sequester Wnt and antagonize Wnt signaling. In this manner, loss of *SFRP/Wif-1* expression can lead to Wnt pathway activation. The genes encoding several of the *SFRPs* and *Wif-1* are epigenetically silenced by DNA methylation in colorectal, lung, bladder, and kidney cancers and lymphocytic leukemia. In prostate cancer, *Wif-1* expression is strongly suppressed. The *SFRP1* gene is also aberrantly hypermethylated in prostate tumors relative to BPH tissue and is partially to completely methylated in several human prostate cancer cell lines. These findings suggest that silencing of genes antagonist to *Wnt* may play a role in prostate cancer development.

The Wnt signaling pathway may interact with other signaling pathways such as the AR-CTNNB1, which in turn can upregulate AR transcriptional activity in an androgen-dependent manner. Subsequently, AR enhances nuclear translocation of CTNNB1. In addition, PI3K/Akt can modulate the activity of CTNNB1 by phosphorylation of CTNNB1 by GSK3B, a substrate of Akt.

MicroRNA and Other Noncoding RNA in Prostate Cancer

Only a small fraction of the transcription output in human genome encodes for proteins. Noncoding RNAs (ncRNAs) are arbitrarily classified into two major classes based on their size: small (microRNA) and long ncRNA (lncRNA).

MicroRNA and Prostate Cancer

Small ncRNAs, exemplified by microRNA (miRNA), are known to regulate diverse biological processes in stem cells,

development, differentiation, metabolism, and disease such as cancer. Several hundred miRNAs have been identified in human cells, and these are transcribed from the genome as long, primary miRNAs ranging in size from hundreds to thousands of nucleotides. miRNAs depend on multiple proteins for their biogenesis and function. Aberrations in any of these proteins will affect miRNA-mediated gene regulation. Expression of Dicer, a key gene in the biosynthesis of miRNA, is upregulated in a significant fraction of prostate cancer and is associated with aggressive cancer features. Knockout of Dicer in the mouse prostate impairs prostate stem cell activity and causes prostate atrophy. Some other components of the miRNA machinery (XPO5, Ago1, Ago2, HSPCA, MOV10, and TNRC6B) are also upregulated in prostate cancer.

Processed miRNAs have been found to regulate gene expression by inhibiting protein translation and/or enhancing the degradation of target gene mRNA with which the miRNAs have imperfect sequence complementarity in the 3′UTR region. It has been predicted that each miRNA can target hundreds of genes, and a third of human protein-coding genes are regulated by miRNA. An increasing body of evidence suggests that miRNAs are involved in the initiation and development of different types of cancers, including prostate cancer.

Expression Signature of miRNA in Prostate Cancer

High-throughput miRNA expression profiling studies have found altered miRNA expression in prostate cancer, providing evidence for the involvement of miRNAs in this disease. miRNAs that have increased expression or amplification in cancer can act as oncogenes to enhance cell proliferation and survival by the inhibition of protein-coding tumor suppressor genes. They are thus called *oncomiRs*. Several such oncomiRs have been identified in prostate cancer cell lines including miR-21, miR-291, miR-221, miR-222, and the well-known miR-17-92 cluster, which contains six members: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. Some common target genes of these oncomiRs include PTEN, BIM, RB1, p21, and p27. On the other hand, miRNAs that normally target and suppress are regarded as tumor suppressor miRNAs (ts-miRNAs). In prostate cancer, the miR-15a/16-1 cluster that targets BCL2 and CCND1 is downregulated in prostate cancer. Forced expression of these miRNAs can lead to suppression of cell proliferation and induction of apoptosis.[10] Other commonly identified tumor suppressor miRNAs include miR-34a/b/c, miR-145, miR-205, and let-7.

miRNA and the AR Signaling Pathway

The AR signaling pathway and miRNAs are engaged in reciprocal regulation via multiple interaction points. miRNA can suppress AR expression through the canonical miRNA pathway or affect AR transcriptional activity by modulating other AR cofactors such as MYC. A systemic analysis of miRNAs identified a number of miRNAs that could suppress AR expression post-transcriptionally by targeting the AR 3′UTR element. Similar to protein coding genes, miRNA genes are also under the regulation of many transcription factors, including AR, which binds to ARE in the promoter of many miRNAs. As the first example, AR can bind to the ARE of miR-21, a prostate oncomiR, and directly regulate its expression as a downstream signaling effector.[11] Several other miRNAs were later found to be also under the regulation of AR, including miR-101, miR-141, miR-27a , miR-32, and miR-148a.

miRNA as Biomarkers for Prostate Cancer Diagnosis

miRNAs derived from cancer cells can exist as circulating extracellular or cell-free RNA and are remarkably stable in body fluids such as plasma/serum. These extracellular miRNAs can be detected and quantified using either PCR-based methods or other high-throughput approaches, including miRNA microarray and next-generation sequencing. A number of studies have performed miRNA profiling in body fluids including serum, plasma, and urine and have identified several miRNAs whose levels can serve as diagnostic and/or prognostic markers for prostate cancer patients.[12,13] Some of the miRNAs that consistently exist in prostate cancer patients across different studies include miR-141 and miR-375.

lncRNA and Prostate Cancer

The longer class of ncRNAs is known as long intergenic RNA (lncRNA), with sizes ranging from a few hundred to thousands of nucleotides. lncRNAs can regulate protein-encoding genes by affecting transcription and chromatin state by mechanisms distinct from those used by small ncRNAs. Thus genetic alterations and aberrant expression of lncRNAs can be a causal factor in disease. Several lncRNAs pertinent to prostate cancer have been identified. A 3.7-kb lncRNA known as PCA3 (DD3) has been mapped to chromosome 9q21-22 and shown to be highly overexpressed in prostate cancer samples. Genetic variation in lncRNAs has been found to affect prostate cancer risk. Microarray profiling of intronic transcripts identified many

ncRNAs expressed in prostate cancer samples, and the expression levels of some correlate with the extent of prostate tumor cell differentiation.[14] By high-throughput RNA sequencing of 102 prostate tissues and cell lines, Prensner and colleagues identified 121 unannotated prostate cancer–associated ncRNA transcripts (PCATs) and have characterized one of them, PCAT-1, as a prostate cancer–specific ncRNA functionally implicated in disease progression.

Given the regulatory role of lncRNA in prostate cancer, their aberrant expression suggests that they may serve as tumor biomarkers. In this regard, Cui and co-workers identified an lncRNA termed *PlncRNA* that expresses higher in prostate cancer cells compared to normal prostate epithelial cells.[15] Knockdown of PlncRNA has an inhibitory effect on prostate cancer cells, potentially through its regulation of AR activity

Epigenetic Alterations in Prostate Cancer

Epigenetic alterations contribute to the malignant transformation and progression of prostate cancer. Figure 38-2 shows the potential contribution of epigenetic events to the development of prostate cancer and its progression to advanced and castration-resistant cancer. Initially DNA methylation was regarded as a new type of promising biomarker for prostate cancer diagnosis and prognosis, and a therapeutic target. Despite intensive research efforts in the past decade, results from clinical studies evaluating DNA methylation as a biomarker have been disappointing, and so far no biomarker has advanced into the clinical arena.

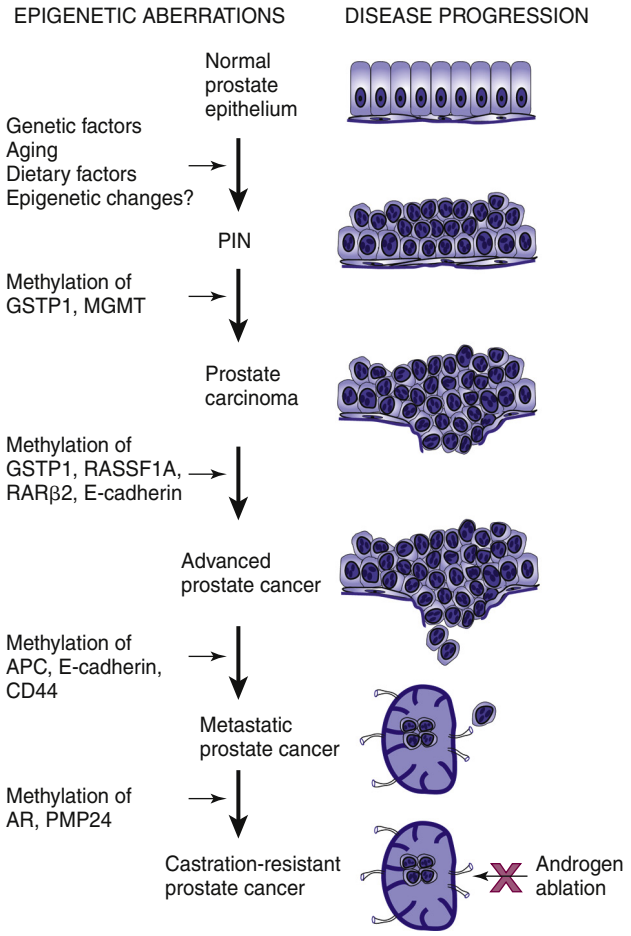


Figure 38-2 Epigenetic alterations in the development of prostate cancer Multiple factors are associated with the development of prostate cancer, including genetic predisposition, environmental factors, diet, ethnicity, and aging. Many of these factors modify the genome through epigenetic effects, and DNA methylation may be an early event causing inactivation of DNA damage repair genes such as *GSTP1* and *MGMT*. Subsequent inactivation of cell-cycle control genes provides a growth advantage leading to locally advanced prostate cancer. Functional loss of genes in the cell adhesion pathway, such as *CD44*, may allow for metastasis. Ultimately, inactivation of AR via DNA hypermethylation allows cancer cells to become androgen insensitive. *From Li LC, Okino ST, Dahiya R. DNA methylation in prostate cancer.* Biochim Biophys Acta. *2004;1704:87-102, with permission.*

Hypermethylation

DNA hypermethylation is one of the most common and best-characterized epigenetic abnormalities in prostate cancer. Genes including classic and putative tumor suppressor genes as well as genes involved in a number of cellular pathways, such as hormonal responses, cancer cell invasion/tumor architecture, cell cycle control, and DNA damage repair, can be hypermethylated in prostate cancer. For many of these genes, promoter hypermethylation is often the mechanism responsible for their functional loss in prostate cancer. Inappropriate silencing of these genes can contribute to cancer initiation, progression, invasion, and metastasis. Some commonly hypermethylated genes in prostate cancer are discussed next (Table 38-2).

DNA Damage-Repair Genes

Antioxidants and DNA repair pathways protect the genome and maintain genome stability during replication or following DNA damage. Hypermethylation of genes important for such processes, such as glutathione *S*-transferase Pi (*GSTP1*) and *O*-6-methylguanine DNA methyltransferase (*MGMT*), has been frequently documented in prostate cancer. *GSTP1*, located at chromosome 11q13, belongs to a supergene family of glutathione *S*-transferases (GSTs) that play an important role in the detoxification of carcinogens and cytotoxic drugs by catalyzing their conjugation to glutathione. *GSTP1* inactivation may lead to increased cell vulnerability to oxidative DNA damage and the accumulation of DNA base adducts, which can precede carcinogenesis.

In prostate cancer, methylation of the *GSTP1* gene promoter is the most frequently detected epigenetic alteration. Elevated CpG methylation has been detected in

Table 38-2 Epigenetic Changes in Prostate Cancer

Gene	Function
Hypermethylation	
GSTP1	Detoxification if electrophilic compounds
RASSF1	Similar to RAS effector proteins
AR	Androgen effects +/–
ESR1, ESR2	Estrogen effects
CCND2, CDKN2A, CDKN1A, SFN	Inhibit cyclin D–associated kinases, other cyclin-dependent kinases
CD44, CDH1, LAMA3, LAMB3	Cell architecture
MGMT	DNA damage repair gene
DAB2IP, EDNRB, RASSF1	Signal transduction
PTGS2	Inflammatory response
Hypomethylation	
CAGE	Novel testis antigen
HPSE	Heparanase
PLAU	Urokinase plasminogen activator
MAGE11	Melanoma antigen gene protein-A11
Histone modification	
VDR	Vitamin D receptor
CPA3	Carboxypeptidase A3
RARB	Retinoic acid receptor β
KLK3	Prostate-specific antigen
DAB2IP	Tumor suppressor

prostate cancer tissues as early as at the stage of atypia and PIN. GSTP1 promoter hypermethylation can also be readily detected in the serum/plasma, urine, and ejaculates of prostate cancer patients with high specificity but unsatisfying sensitivity. Current findings from a large number of studies do not support the feasibility of using GSTP1 as an independent tumor biomarker, but it may complement prostate-specific antigen (PSA) screening for prostate cancer diagnosis.

Hormone Receptors

AR mediates testosterone and dihydrotestosterone activity, which is essential for the development and maturation of the prostate gland and prostate cancer. Most prostate cancer is initially androgen dependent, but eventually becomes androgen independent after androgen-deprivation therapy. Androgen-independent prostate cancers are characterized by a heterogeneous loss of *AR* expression. Genetic alterations that alter the sensitivity of the receptor to androgen, such as *AR* gene mutation and, more commonly, amplification without loss of *AR* expression, are thought to play key roles in the development of androgen-independent prostate cancer. The prostate expresses two types of estrogen receptors (ERs): ERα (ESR1) and ERβ (ESR2), whose functional role in the prostate and prostate cancer remains controversial. Hypermethylation of ERs leading to decreased expression increases with aging and can contribute to tumor progression in prostate cancer patients.

Cell Cycle Control Genes

An important characteristic of tumor cells is unbalanced proliferation due to impaired regulation of the cell cycle. The multiple checkpoints that control the cell cycle include the retinoblastoma protein, cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). CDKIs are potential tumor suppressor genes that act as molecular brakes on cell cycle progression. Failure of cell cycle arrest due to alterations in CDKI expression has been implicated in prostate cancer. CDKIs are grouped into two families: the INK4 family and the CIP/KIP (kinase inhibitor protein) family. The INK4 family includes CDKN2A (p16), CDKN2B (p15), CDKN2C (p18), and CDKN2D (p19) and inhibits cyclin D–associated kinases (CDK4 and CDK6). The CIP/KIP family, which includes CDKNIA (p21), CDKN1B (p27), and CDKN1C (p57), inhibits most CDKs.

CDKN2A can be inactivated in prostate cancer by a variety of mechanisms, including deletion, point mutation, and hypermethylation. Methylation-mediated inactivation of the *CDKN2A* gene has been reported in prostate cancer cell lines and tissues at a very low frequency (0% to 16%). Inactivation of other cell cycle genes such as CDKN2B, CDKN1A, and CDKN1B by hypermethylation is rare in prostate cancer.

The Ras-association domain family-1 gene (*RASSF1*) is located at 3p21.3 and encodes a protein similar to the RAS effector proteins. A tumor suppressor role has been proposed for *RASSF1*. *RASSF1* promoter methylation is a common event in prostate cancer and high-grade PIN, occurring in 54% to 96% of tumors, and increases with higher Gleason scores.

Tumor Invasion and Tumor Architecture Genes

The cadherin-catenin adhesion system is critical for the preservation of normal tissue architecture and is regulated by a family of proteins collectively termed *cell adhesion molecules* (CAMs). Decreased expression of CDH1 and other CAMs

has been reported to have prognostic significance in various human cancers, including prostate cancer. In prostate cancer, expression of CDH1 is markedly suppressed and its promoter is methylated to varying degrees. In addition, methylation of the CDH1 promoter is increased in advanced prostate cancer, making it a potential biomarker for cancer progression.

CD44 is an integral membrane protein involved in matrix adhesion and signal transduction. Loss of CD44 expression correlates with methylation of its gene promoter in prostate cancer and is associated with stage and prognosis. Other genes involved in the cadherin-catenin adhesion system have also shown methylation-mediated inactivation in prostate cancer, such as H-cadherin, adenomatous polyposis coli (APC), caveolin-1 (CAV1), laminin α-3 (*LAMA3*), laminin β-3 (*LAMB3*), and laminin γ-2 (*LAMC2*).

Other Putative Tumor Suppressor Genes

Other possible tumor suppressor genes that are subject to epigenetic inactivation in prostate cancer include *KAI1* (a prostate-specific tumor metastasis suppressor gene), inhibin-α (a member of the TGF-β family of growth and differentiation factors), and *DAB2IP*, a novel GTPase-activating protein for modulating the Ras-mediated signal pathway.[16] Recent high-throughput DNA methylation profiling in prostate cancer cell lines and tissues unveiled methylated genes such as SLC15A3, KRT7, TACSTD2, GADD45b[17] OXD3 and BMP7[18]PR83, ADCY4, LOC63928, and D4S234E;[19] however, these genome-wide analyses seem not to have yielded consistent information identifying new prostate cancer biomarkers.

Hypomethylation

Both global and gene-specific hypomethylation have been implicated in human malignancy. The *PLAU* gene encodes urokinase plasminogen activator and is highly expressed in most prostate cancer tissues and invasive prostate cancer cell lines. DNA methylation and gene amplification may participate in the regulation of the *PLAU* gene in prostate cancer. Hypomethylation of the PLAU promoter is associated with increased expression in hormone-independent prostate cancer cells, higher invasive capacity in vitro, and increased tumorigenesis in vivo. Other hypomethylated genes in prostate cancer include *CAGE*, a novel cancer/testis antigen gene; heparanase (*HPSE*), *CYP1B1*, and Melanoma antigen gene protein-A11 (MAGE-11). *HPSE*, an endo-β-*D*-glucuronidase, and *CYP1B1* are overexpressed and substantially hypomethylated in prostate cancer compared with benign prostatic hyperplasia samples. Aberrant hypomethylation of repetitive DNA elements such as LINE-1 and ncRNA such as XIST also occurs in prostate cancer.

Histone Modification

DNA is organized into a nucleoprotein complex termed *chromatin*. The basic chromatin unit is the nucleosome, which is composed of 146 bp of DNA wrapped around four pairs of histone proteins. The N-terminal tails of histones are positioned outside the nucleosome core and are thus susceptible to covalent modifications including acetylation and methylation. Acetylation and deacetylation of histone tails are catalyzed by histone acetyltransferase (HATs) and deacetylases (HDACs), respectively. Through histone acetylation, HATs have been shown to increase the activity of several transcription factors, including nuclear hormone receptors, which facilitate promoter access to the transcriptional machinery. Conversely, HDACs cause histone deacetylation, which is associated with transcriptional repression. Histone methylation is facilitated by histone methyltransferases (HMTs), which use *S*-adenosylmethionine as a methyl donor group to the lysine and arginine residues of histone protein pairs H3 and H4. Like histone acetylation, histone methylation is reversible and is facilitated by at least two enzymes: lysine-specific demethylase1 (LSD1) and JmjC domain-containing histone demethylase1 (JHDM1).

The expression of many genes is aberrantly regulated in prostate cancer through histone modification. Tumor-specific alterations in the enzymes that modify histone states can alter global histone modification profiles. For example, MLL2 encodes an H3K4-specific histone methyltransferase that is recurrently mutated in multiple cancers including prostate cancer. Also, the loss of acetylation of H3 and H4 resulting from increased HDAC activity may also be of importance in prostate cancer. Treatment of prostate cancer cells with HDAC inhibitors increased the expression of specific genes such as insulin-like growth factor binding protein-3 and carboxypeptidase A3 (CPA3), thereby suggesting a role for histone acetylation in aberrant gene regulation.

Polycomb Group Transcriptional Repression

The Polycomb group (PcG) proteins are developmental regulators that silence chromatin through H3K27 methylation. Enhancer of Zeste 2 (*EZH2*) and SUZ12 are members of the PcG proteins that are overexpressed in prostate cancer and are highly associated with tumor aggressiveness.

Other studies reveal that *EZH2* is overexpressed and associated with aggressiveness in cutaneous melanoma, endometrial cancer, bladder cancer, and breast cancer. In addition, other PcG proteins, *BMI1* and *RING1*, are also overexpressed in aggressive prostate cancers. The *EZH2* complex silences gene expression by catalyzing H3K27 methylation to generate an inaccessible, heterochromatic chromatin configuration. In addition, *EZH2* was found to control DNA methylation through direct physical contact with DNA methyltransferase. It is unclear how *EZH2* is overexpressed in prostate cancer. EZH2 overexpression in prostate cancer causes the silencing of developmental regulators and tumor suppressor genes such as ADRB2, CDH1, DAB2IP, SNCA, and SOCS via histone methylation, conferring on cancer cells a stem cell–like epigenetic state, because PcG is a stem cell–specific marker including cancer stem cells and plays an important role in maintaining the undifferentiated state of embryonic stem (ES) cells. Pharmacologic disruption of polycomb repressive complex 2 has been shown to inhibit prostate tumorigenicity and tumor progression in animal models of prostate cancer.

PSA

The PSA (KLK3) gene contains an androgen receptor response element in its 5′ regulatory region. Methylation of H3K4 is associated with transcriptional inactivation of the PSA gene in the prostate cancer cell line LNCaP, and transcription of the PSA gene is accompanied by rapid decreases in di- and trimethylated H3 at lysine 4. In addition, a lysine-specific demethylase (LSD1) has been found to interact with the androgen receptor to stimulate the AR-dependent transcription of PSA in LNCaP cells by removing the methyl group at H3K9. An inhibitor of LSD1, pargyline, can block AR-dependent transcription by blocking histone demethylation. The net effect of methylation on the PSA gene needs to be carefully considered, however, because PSA is a marker of disease progression rather than a causal factor.

Aberrant Translational Control in Prostate Cancer Etiology and Progression

In addition to the genomic and transcriptional alterations that drive cancer initiation and progression, there is an emerging appreciation for how altered protein synthesis may have a direct causal role in cancer etiology. The understanding of translational control has undergone a paradigm shift towards a greater appreciation for specificity in this step of gene expression regulation. For example, genes that encode distinct factors involved in translation initiation, the first and most highly regulated step of protein synthesis, are often found aberrantly expressed in human cancers.[19a-19c] Furthermore, translation of mRNA networks involved in tumor suppression and oncogenic transformation is controlled by the presence of regulatory elements in their 5′- and 3′UTRs such as internal ribosome entry sites (IRESes), structured RNA sequences, RNA binding protein domains, and miRNA binding sites. Most importantly, activity of an entire repertoire of translational components is controlled by oncogenic signal transduction pathways such as RAS, PI3K-AKT-mTOR, and MYC, which are master regulators of protein synthesis. These oncogenic signaling pathways are commonly deregulated in human prostate cancer and have been shown to promote cancer initiation and progression.[19d-19h] A striking example is the convergence of RAS, PI3K-AKT-mTOR, and MYC pathways on translation initiation. In this context, these pathways share a common regulatory node: oncogenes[19i] and translational initiation factor eIF4E, which controls global protein synthesis as well as the translation of specific mRNA targets.[19b,19g-19l] eIF4E is the best characterized and rate-limiting factor of the eIF4F translation initiation complex. The activity of eIF4E is negatively regulated by the tumor suppressor eIF4E binding proteins (4EBPs), which are phosphorylated and inhibited by the mTOR kinase.

Deregulation of the 4EBP/eIF4E axis has been linked to prostate cancer initiation and progression. In particular, it has been shown that eIF4E phosphorylation, which directs eIF4E activity, is necessary for tumorigenesis in a mouse model of prostate cancer driven by PI3K-AKT-mTOR hyperactivation.[20] Restraining the oncogenic activity of eIF4E downstream of hyperactive mTOR to normal levels inhibits tumor progression and leads to overall increased survival rates.[21] One of the molecular mechanisms underlying eIF4E's oncogenic activity is its ability to upregulate the translation of pro-survival factors. For example, distinct mRNAs with highly structured 5′UTRs (which is an obstacle for translation initiation), such as the anti-apoptotic protein Mcl-1, are more efficiently translated on eIF4E hyperactivation.[19b] Mechanistically, eIF4E recruits the eIF4A helicase to unwind these 5′UTR structured elements, leading to the translational upregulation of specific mRNAs such as Mcl-1.[22,23] Thus, eIF4E hyperactivation provides a survival advantage for cancer cells.

Genetic and pharmacological inhibition of the 4EBP1/eIF4E axis downstream of oncogenic mTOR also significantly inhibits the invasive and metastatic potential of prostate cancer.[19l] The molecular mechanism by which hyperactive eIF4E exploits specific cellular processes to drive cancer progression is also mediated through the selective translation of distinct nodes of mRNAs. In this context, the translation of a novel signature of metastasis-associated

mRNAs is found to be upregulated during prostate cancer invasion. This cancer cell invasion network includes the intermediate filament protein vimentin,[24] whose expression is increased during cancer progression; CD44, an antigen involved in cell migration that is also associated with cancer metastasis;[25] metastasis associated protein 1 (MTA1), which promotes neoangiogenesis in metastatic forms of cancer;[26,27] and Y-box binding protein 1 (YB-1).[28] The eIF4E-mediated translation of this invasion signature is dependent on a newly identified regulatory element termed a pyrimidine-rich translational element (PRTE) in the 5'UTR of these mRNAs. Importantly, a new and potent class of compounds that blocks mTOR oncogenic activity through inhibition of eIF4E (known as mTOR ATP site inhibitors) decreases the expression of the invasion signature and demonstrates therapeutic efficacy during all stages of prostate cancer progression and metastasis. Additional mRNA targets that direct cell invasion are also controlled by eIF4E including matrix metalloproteinase 9 (MMP9), an enzyme that aids breakdown of extracellular matrix (ECM), and heparanase, which degrades the interior lining of blood vessels. Thus, deregulation of translational control represents a highly specific mechanism for targeting critical nodes of gene expression that can steer prostate epithelial cells towards transformation and cancer progression.[19l,29,30] Interestingly, recent studies have uncovered that YB-1 in turn promotes cap-independent modes of translation for other mRNAs involved in metastasis.

Components of the translational machinery are increasingly being recognized as potential biomarkers of disease progression and attractive therapeutic targets in prostate cancer. eIF4E overexpression is common in multiple cancer types, including malignancies of the prostate, breast, stomach, colon, lung, skin, and the hematopoietic system.[30] In prostate cancer, eIF4E protein and phosphorylation levels as well as 4EBP1 phosphorylation status correlate positively with Gleason grade.[31,32] Furthermore, in postprostatectomy prostate cancer patients, elevated eIF4E expression and increased 4EBP1 phosphorylation are predictive of worse overall survival.[32] Beyond the translational machinery itself, specific downstream mRNA targets of translation initiation factors, including the eIF4E-directed invasion signature described earlier, are also candidate biomarkers for prostate cancer progression. For example, prostate cancer patients exhibit stepwise increases in YB-1 expression at every stage of the disease from normal prostate to castration-resistant prostate cancer.[19l] Furthermore, the matrix metalloproteinase, MMP3, which is a translationally regulated mRNA responsive to eIF4E phosphorylation, is highly expressed in prostate cancer.[31] Other initiation factors are also emerging as biomarkers, including eIF3H, a component of the translation pre-initiation complex that is highly overexpressed at the protein level in human prostate cancer.[33] In addition to the translation initiation machinery, there is evidence suggesting that the abundance of rRNA (ribosomal RNA), a critical component of the ribosome, increases with advanced prostate cancer.[34,35] Moreover, the gene that encodes a key enzyme that modifies specific rRNA nucleotides, known as *DKC1*, is significantly overexpressed in prostate cancers, particularly in patients with aggressive disease. Therefore, multiple components of the translation machinery as well as downstream mRNA targets may have significant predictive power that could help delineate between prostate cancer patients with indolent and those with aggressive forms of the disease. Most importantly, next-generation therapeutics have shown great promise in preclinical and Phase I clinical trials at targeting the oncogenic translation machinery in prostate cancer.[37,38]

Future Directions

The search for new biomarkers is under way to aid in the diagnosis, prognosis, and decision-making process of men with prostate cancer. Because of the multitude of mechanisms of carcinogenesis, many approaches can be taken to accomplish these aims. Because epigenetic events are theoretically reversible, novel therapies that target hyper- or hypomethylated genes or histone acetylation implicated in prostate cancer aggressiveness and progression could bring new hope to patients with metastatic disease. Although the past two decades have been largely dedicated to the use and importance of PSA, the future of prostate cancer detection and treatment will be refined by discoveries in molecular biology.

Key References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
2. Baade PD, Coory MD, Aitken JF. International trends in prostate-cancer mortality: the decrease is continuing and spreading. *Cancer Causes Control*. 2004;15:237-241.
3. Andriole GL, et al. Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med*. 2009;360:1310-1319.
4. Schroder FH, et al. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med*. 2009;360:1320-1328.
5. Arora R, et al. Heterogeneity of Gleason grade in multifocal adenocarcinoma of the prostate. *Cancer*. 2004;100:2362-2366.
6. Johns LE, Houlston RS. A systematic review and meta-analysis of familial prostate cancer risk. *29BJU Int*. 2003;91:789-794.

7. Grasso CS, et al. The mutational landscape of lethal castration-resistant prostate cancer. *Nature*. 2012;487:239-243.
8. Taylor BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18:11-22.
9. Tomlins SA, et al. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. *Cancer Res*. 2006;66:3396-3400.
10. Casanova-Salas I, et al. miRNAs as biomarkers in prostate cancer. *Clin Transl Oncol*. 2012;14:803-811.
11. Ribas J, et al. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Res*. 2009;69:7165-7169.
12. Moltzahn F, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res*. 2011;71:550-560.
13. Kuner R, et al. microRNA biomarkers in body fluids of prostate cancer patients. *Methods*. 2013;59:132-137.
14. Reis EM, et al. Antisense intronic non-coding RNA levels correlate to the degree of tumor differentiation in prostate cancer. *Oncogene*. 2004;23:6684-6692.
15. Cui Z, et al. The prostate cancer-up-regulated long noncoding RNA PlncRNA-1 modulates apoptosis and proliferation through reciprocal regulation of androgen receptor. *Urol Oncol*. 2013 Oct;31(7):1117-1123.
16. Chen G, et al. Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer*. 2004;101:1345-1356.
17. Ibragimova I, et al. Global reactivation of epigenetically silenced genes in prostate cancer. *Cancer Prev Res (Phila)*. 2010;3:1084-1092.
18. Kron K, et al. Discovery of novel hypermethylated genes in prostate cancer using genomic CpG island microarrays. *PLoS One*. 2009;4:e4830.
19. Kim SJ, et al. Genome-wide methylation analysis identifies involvement of TNF-alpha mediated cancer pathways in prostate cancer. *Cancer Lett*. 2011;302:47-53.
20. Furic L, et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proc Natl Acad Sci U S A*. 2010;107:14134-14139.
21. Etzioni R, et al. The prostate cancer conundrum revisited: treatment changes and prostate cancer mortality declines. *Cancer*. 2012;118:5955-5963.
29. Konicek BW, Dumstorf CA, Graff JR. Targeting the eIF4F translation initiation complex for cancer therapy. *Cell Cycle*. 2008;7:2466-2471, doi:6464 [pii].
30. Silvera D, Formenti S, Schneider R. Translational control in cancer. *Nature reviews cancer*. 2010;19:254-266-266, doi:10.1038/nrc2824.
37. Ruggero D. Translational control in cancer etiology. *Cold Spring Harb Perspect Biol*. 2013;5:a012336.
38. Malina A, Mills JR, Pelletier J. Emerging therapeutics targeting mRNA translation. *Cold Spring Harb Perspect Biol*. 2012;4:a012377.

This page intentionally left blank

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
2. Baade PD, Coory MD, Aitken JF. International trends in prostate-cancer mortality: the decrease is continuing and spreading. *Cancer Causes Control*. 2004;15:237-241.
3. Andriole GL, et al. Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med*. 2009;360:1310-1319.
4. Schroder FH, et al. Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med*. 2009;360:1320-1328.
5. Arora R, et al. Heterogeneity of Gleason grade in multifocal adenocarcinoma of the prostate. *Cancer*. 2004;100:2362-2366.
6. Johns LE, Houlston RS. A systematic review and meta-analysis of familial prostate cancer risk. *29BJU Int*. 2003;91:789-794.
7. Grasso CS, et al. The mutational landscape of lethal castration-resistant prostate cancer. *Nature*. 2012;487:239-243.
8. Taylor BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18:11-22.
9. Tomlins SA, et al. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. *Cancer Res*. 2006;66:3396-3400.
10. Casanova-Salas I, et al. miRNAs as biomarkers in prostate cancer. *Clin Transl Oncol*. 2012;14:803-811.
11. Ribas J, et al. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. *Cancer Res*. 2009;69:7165-7169.
12. Moltzahn F, et al. Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. *Cancer Res*. 2011;71:550-560.
13. Kuner R, et al. microRNA biomarkers in body fluids of prostate cancer patients. *Methods*. 2013;59:132-137.
14. Reis EM, et al. Antisense intronic non-coding RNA levels correlate to the degree of tumor differentiation in prostate cancer. *Oncogene*. 2004;23:6684-6692.
15. Cui Z, et al. The prostate cancer-up-regulated long noncoding RNA PlncRNA-1 modulates apoptosis and proliferation through reciprocal regulation of androgen receptor. *Urol Oncol*. 2013;31(7):1117-1123.
16. Chen G, et al. Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. *Cancer*. 2004;101:1345-1356.
17. Ibragimova I, et al. Global reactivation of epigenetically silenced genes in prostate cancer. *Cancer Prev Res (Phila)*. 2010;3:1084-1092.
18. Kron K, et al. Discovery of novel hypermethylated genes in prostate cancer using genomic CpG island microarrays. *PLoS One*. 2009;4:e4830.
19. Kim SJ, et al. Genome-wide methylation analysis identifies involvement of TNF-alpha mediated cancer pathways in prostate cancer. *Cancer Lett*. 2011;302:47-53.

19a. Alliouachene S, et al. Constitutively active Akt1 expression in mouse pancreas requires S6 kinase 1 for insulinoma formation. *J Clin Invest*. 2008;118:3629-3638, doi:10.1172/JCI35237.

19b. Hsieh AC, et al. Genetic Dissection of the Oncogenic mTOR Pathway Reveals Druggable Addiction to Translational Control via 4EBP-eIF4E. *Cancer Cell*. 2010;17:249-261, doi:10.1016/j.ccr.2010.01.021.

19c. Janes MR, et al. Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor. *Nat Med*. 16, 205–213, doi:nm.2091 [pii].

19d. Edwards J, Krishna NS, Witton CJ, Batlett JM. Gene amplifications associated with the development of hormone-resistant prostate cancer. *Clin Cancer Res*. 2003;9:5271-5281.

19e. Taylor BS, et al. Integrative genomic profiling of human prostate cancer. *Cancer cell* 18, 11–22, doi:S1535-6108(10)00238-2 [pii].10.1016/j.ccr.2010.05.026.

19f. Brown EJ, et al. Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature*. 1995;377:441-446, doi:10.1038/377441a0.

19g. Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes & development*. 1998;12:502-513.

19h. Jones RM, et al. An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc. *Mol Cell Biol*. 1996;16:4754-4764.

19i. Ruggero D, et al. The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat Med*. 2004;10(5):484-486.

19j. Lin CJ, et al. Targeting synthetic lethal interactions between Myc and the eIF4F complex impedes tumorigenesis. *Cell reports*. 2012;1:325-333, doi:10.1016/j.celrep.2012.02.010.

19k. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *The EMBO journal*. 1997;16:1909-1920, doi:10.1093/emboj/16.8.1909.

19l. Hsieh AC, et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature*. 2012, doi:10.1038/nature10912.

20. Furic L, et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proc Natl Acad Sci U S A*. 2010;107:14134-14139.
21. Etzioni R, et al. The prostate cancer conundrum revisited: treatment changes and prostate cancer mortality declines. *Cancer*. 2012;118:5955-5963.
22. Haghighat A, Sonenberg N. eIF4G dramatically enhances the binding of eIF4E to the mRNA 5′-cap structure. *J Biol Chem*. 1997;272(35):21677-21680.
23. Rogers Jr. GW, Richter NJ, Merrick WC. Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. 1999;274(18): 12236-12244.
24. Lahat G, et al. Vimentin is a novel anti-cancer therapeutic target; insights from in vitro and in vivo mice xenograft studies. *PloS one* 5. e10105, doi:10.1371/journal.pone.0010105.
25. Liu C, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nature. medicine* 17. 211–215, doi:nm.2284 [pii]10.1038/nm.2284.
26. Hofer MD, et al. The role of metastasis-associated protein 1 in prostate cancer progression. *Cancer research*. 2004;64:825-829.
27. Yoo YG, Kong G, Lee MO. Metastasis-associated protein 1 enhances stability of hypoxia-inducible factor-1alpha protein by recruiting histone deacetylase 1. *The EMBO journal* 25. 1231–1241, doi:7601025 [pii].
28. Evdokimova V, et al. Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. *Cancer Cell* 15. 402–415, doi:S1535-6108(09)00086-5 [pii].
29. Konicek BW, Dumstorf CA, Graff JR. Targeting the eIF4F translation initiation complex for cancer therapy. *Cell Cycle*. 2008;7:2466-2471, doi:6464 [pii].
30. Silvera D, Formenti S, Schneider R. Translational control in cancer. *Nature reviews. Cancer*. 2010;10:254-266, doi:10.1038/nrc2824.
31. Furic L, et al. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proc Natl Acad Sci U S A*. 2010;107(32):14134-14139.
32. Graff JR, et al. eIF4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival. *Cancer research*. 2009;69:3866-3873, doi:0008-5472.CAN-08-3472 [pii]10.1158/0008-5472.CAN-08-3472.
33. Zhang L, Smit-McBride Z, Pan X, Rheinhardt J, Hershey JW. An oncogenic role for the phosphorylated h-subunit of human translation initiation factor eIF3. *The Journal of biological chemistry*. 2008;283:24047-24060, doi:10.1074/jbc.M800956200.
34. Ibaragi S, et al. Angiogenin-stimulated rRNA transcription is essential for initiation and survival of AKT-induced prostate intraepithelial neoplasia. *Molecular cancer research: MCR*. 2009;7:415-424, doi:10.1158/1541-7786.MCR-08-0137.
35. Uemura M, et al. Overexpression of ribosomal RNA in prostate cancer is common but not linked to rDNA promoter hypomethylation. *Oncogene*. 2012;32:1254-1263, doi:10.1038/onc.2011.319.
36. Sieron P, et al. DKC1 overexpression associated with prostate cancer progression. *Br J Cancer*. 2009;101:1410-1416, doi:10.1038/sj.bjc.6605299.
37. Ruggero D. Translational control in cancer etiology. *Cold Spring Harb Perspect Biol*. 2013;5:a012336.
38. Malina A, Mills JR, Pelletier J. Emerging therapeutics targeting mRNA translation. *Cold Spring Harb Perspect Biol*. 2012;4:a012377.

Sabina Signoretti, Toni K. Choueiri, and William G. Kaelin, Jr.

39

Molecular Abnormalities in Kidney Cancer

Kidney cancer is one of the 10 most common cancers in the United States in both men and women. A number of subtypes of kidney cancer exist. Historically these subtypes have been recognized based on their histological appearance, but it is also now known that each subtype has characteristic molecular abnormalities as well. Germline and somatic genetic studies have helped identify tumor suppressor genes and proto-oncogenes that play roles in kidney cancer pathogenesis.

The principal objectives of this chapter are to review the following: (1) the epidemiology of kidney cancer, (2) the histopathological subtypes of kidney cancer, (3) the genetic alterations linked to hereditary kidney cancer, (4) the molecular pathogenesis of kidney cancer with a focus on clear cell renal carcinoma, and (5) the potential utility of this information for the early detection and management of kidney cancer.

Epidemiology

Kidney cancer accounts for more than 3% of all malignant diseases. It occurs more frequently in men than in women (M/F ratio = 1.7:3.4), with highest incidence in the sixth and seventh decades. In the United States, incidence rates tend to be higher in African-Americans compared to Caucasians.[1] In the past 65 years, the incidence of kidney cancer has increased steadily at about 2.5% per year. Most kidney cancers (more than 90%) arise in the kidney parenchyma; the remainder originate from the renal pelvis. Renal cell carcinoma (RCC) is the most common form of cancer of the kidney parenchyma that occurs in adults.[1] Most RCCs, in turn, are clear cell renal carcinomas (ccRCC).

There is strong evidence that genetic factors are linked with an increased risk of RCC. Although most renal cancers are sporadic, a small percentage of patients (2% to 5%) have an inherited predisposition to the disease[2] (Table 39-1). In further support of the notion that genetic factors may predispose to renal cancer, a genome-wide association study of RCC recently identified two susceptibility loci, one linked to *EPAS1* on 2p21 that encodes hypoxia inducible factor-2 alpha (HIF2α) and another linked to a HIF-binding site near the *Cyclin D1* locus.[3,4]

Kidney Cancer Histopathology

It is widely accepted that RCC is not a single disease but includes different types of epithelial malignancies of the kidney that are characterized by different histopathologic features and also display different genetic alterations and clinical behavior.[5,6] The current World Health Organization (WHO) classification of kidney tumors is based on both histopathologic and molecular criteria and recognizes 10 major types of RCCs (Table 39-2). Clear cell RCC (ccRCC) is the most common renal cancer type and accounts for 70% to 80% of all cases.

Papillary RCC (pRCC) represents approximately 10% of RCC cases. Chromophobe RCC (chRCC; about 5% of all cases) is grossly a well-circumscribed tumor with a gray to brown cut surface. Microscopically, it consists of tumor cells with pale cytoplasm and prominent cell membrane.

Xp11 translocation RCCs, which bear gene fusions involving the *TFE3* gene at Xp11.2, represent the vast majority of translocation RCCs (tRCCs). Histologically, tRCC usually shows a papillary pattern of growth and the presence of cells with abundant clear or eosinophilic cytoplasm.

Hereditary Kidney Cancer Syndromes and Molecular Pathways of Kidney Carcinogenesis

Studying rare families that are predisposed to cancer because of highly penetrant germline mutations has often led to the

Table 39-1 Inherited Renal Cancer

Syndrome/Disease	Kidney Cancer Type(s)	Extrarenal Manifestations	Gene (Locus)
von Hippel-Lindau (VHL)	Clear cell RCC	CNS and retinal hemangioblastomas, pancreatic cysts and neuroendocrine tumors, phechromocytomas, endolymphatic sac tumors	*VHL (3p25)*
Hereditary papillary renal carcinoma (HPRC)	Papillary RCC, type 1	—	*MET (7q31)*
Hereditary leiomyomatosis and renal cell carcinoma (HLRCC)	Papillary RCC, type 2	Leiomyomas, leiomyosarcomas	*FH (1q42)*
Birt-Hogg-Dubé (BHD)	Chromophobe RCC, oncocytic tumors, clear cell RCC (rare)	Cutaneous lesions (fibrofolliculomas), pulmonary cysts, and spontaneous pneumothorax	*FLCN (17p11)*
Familial clear cell renal cell carcinoma (FCRC)	Clear cell RCC	—	*?*
Tuberous sclerosis (TS)	Angiomyolipoma, clear cell RCC (rare)	Cortical tubers, subependymal giant cell astrocytomas, cutaneous lesions, cardiac rhabdomyomas, pulmonary cysts, retinal hamartomas	*TSC 1 (9q34) TSC 2 (16p13)*
Constitutional chromosome 3 translocation	Clear cell RCC	—	*?*
Hyperparathyroidism–jaw tumor syndrome (HPT-JT)	Mixed epithelial-stromal tumors, Wilms tumor (rare)	Parathyroid adenomas and carcinomas, ossifying fibromas of the jaw	*CDC73 (1q25)*
Familial papillary thyroid carcinoma (fPTC)	Papillary RCC	Papillary thyroid carcinomas, multinodular goiters	*? (1q21)*
Pheochromocytoma/paraganglioma syndrome type 4 (PGL4)	Various histological types have been reported	Pheochromocytomas and paragangliomas, gastrointestinal stromal tumors	*SDHB (1p36)*

Table 39-2 2004 WHO Classification Of RCC

Clear cell renal cell carcinoma
Multilocular clear cell renal cell carcinoma
Papillary renal cell carcinoma
Chromophobe renal cell carcinoma
Carcinoma of the collecting ducts of Bellini
Renal medullary carcinoma
Xp11 translocation carcinomas
Carcinoma associated with neuroblastoma
Mucinous tubular and spindle cell carcinoma
Renal cell carcinoma, unclassified

discoveries of genes and pathways that play much broader roles in human cancer. In particular, the genes linked to these particular syndromes are often somatically mutated in the sporadic counterparts of the tumors that define these syndromes.

Approximately 2% to 5% of kidney cancers are linked to a recognized cancer predisposition syndrome, and there is at least one such syndrome for each of the major histological kidney cancer subtypes (see Table 39-1). Germline *VHL* mutations cause VHL disease, in which ccRCC is a dominant feature, whereas germline *c-Met* and *fumarate hydratase* mutations are linked to hereditary Type 1 and Type 2 pRCC, respectively. Germline *FLCN* mutations cause familial oncocytomas together with an increased risk of renal cell carcinoma (especially chRCC).

von Hippel-Lindau Disease

von Hippel-Lindau disease is an autosomal dominant disorder caused by germline loss-of-function mutations of the von Hippel-Lindau tumor suppressor gene (*VHL*), which is located on chromosome 3p25, and affects about 1 in 35,000 people.[7] Most VHL patients, however, will exhibit some manifestations of their disease by early adulthood. Hemangioblastomas and kidney cancer are the two leading causes of death in this population. All patients with a clinical diagnosis of VHL disease harbor a *VHL* mutation. A number of genotype-phenotype correlations have been described in this setting, however, with respect to the risk of developing specific tumors such as kidney cancer and pheochromocytoma. Type 1 VHL disease is associated with low risk of pheochromocytoma and Type 2 with a high risk of pheochromocytoma. Type 2 disease is subdivided into Type 2A (low risk of kidney cancer), Type 2B (high risk of kidney cancer), and Type 2C (pheochromocytoma only).

Somatic *VHL* Mutations and Hypermethylation in Sporadic Clear Cell Renal Carcinoma

Of sporadic ccRCCs, 40% to 80% have inactivated both *VHL* alleles (maternal and paternal), most often as a result of a

Table 39-3 Somatic Genetic Alterations in Sporadic Renal Carcinomas

Histologic Type	Genetic Alterations	Frequency of Alterations
Clear cell RCC	Mutations	
	VHL	42%-82%
	PBRM1	~40%
	BAP1	~15%
	Copy number alterations	
	3p deletion	86%-98%
	5q gain	40%-69%
	14q deletion	20%-42%
Papillary RCC	Mutations	
	MET	0%-13%
	Copy number alterations	
	Trisomy 7, 17	61%-89%
Chromophobe RCC	Copy number alterations	
	Monosomy 1, 2, 6, 10, 13, 17	68%-100%
Translocation RCC	Translocations	
	Translocations involving Xp11.2 (*TFE3*)	>90%
	t(6;11)(p21;q12) (*Alpha-TFEB*)	Rare
Medullary carcinoma	Translocations	
	t(2;10)(p23;q22) (*VCL-ALK*)	?

somatic intragenic mutation affecting one VHL allele and a large chromosome 3p somatic deletion spanning the other (Table 39-3). In short, the *VHL* tumor suppressor gene, like the *RB1* tumor suppressor gene, conforms to the Knudson two-hit model of carcinogenesis. An additional 10% to 20% of clear cell renal carcinomas have not sustained *VHL* mutations but fail to produce normal levels of the *VHL* mRNA (and hence protein) because the maternal and paternal *VHL* loci are hypermethylated. Some other clear cell carcinomas display a transcriptional signature suggestive of *VHL* inactivation despite an apparent absence of *VHL* mutations or hypermethylation.

VHL Protein Function

The *VHL* gene encodes two proteins by virtue of two alternative, in-frame, translation initiation codons.[8-10] Both protein isoforms behave similarly in most (but not all) assays performed to date, and the *VHL* mutations linked to kidney cancer almost invariably affect both isoforms. pVHL dynamically shuttles between the cytoplasm and the nucleus, although at steady state most of the protein is cytoplasmic. pVHL is a multifunctional protein whose function relates to its role as the substrate recognition module of an E3 ubiquitin ligase complex that also contains elongin B, elongin C, Cul2, and Rbx1[11] (Figure 39-1). The crystal structure of pVHL bound to elongin B and elongin C reveals that pVHL has two subdomains, called the alpha domain and the beta domain, which are hotspots for mutations in VHL disease and which are, directly or indirectly, affected by kidney cancer–associated mutations. A number of putative pVHL substrates have been identified, including members of the HIF transcription factor family.

The HIF Transcription Factor

HIF (hypoxia-inducible factor) is a heterodimeric transcription factor consisting of an alpha subunit and a beta subunit, both of which are members of the basic Helix-Loop-Helix-PAS domain family.[12,13] There are three human HIFα (HIF1α, HIF2α, HIF3α) and three human HIFβ (often referred to as ARNTs for aryl hydrocarbon receptor nuclear translocators) family members.

In the presence of oxygen, HIFα is hydroxylated on one (or both) of two prolyl sites by members of the EglN (also called PHD) prolyl hydroxylase family[14] (see Figure 39-1). Hydroxylation of either of these sites generates a binding site for pVHL, which then directs the polyubiquitylation of the HIFα subunit. Once ubiquitylated, HIFα is then degraded by the proteasome. Under low-oxygen conditions EglN activity is impaired because these enzymes require oxygen, in addition to 2-oxoglutarate and reduced iron, in order to function. This leads to stabilization and accumulation of HIFα, which then binds to HIFβ, translocates to the nucleus, and activates 100 to 200 genes that contain hypoxia-response elements and promote adaptation to hypoxia. In contrast to HIF1α and HIF2α, most of the HIF3α isoforms detected in cells, which arise because of alternative splicing of the HIF3α mRNA, lack transactivation capability and may, in fact, serve to blunt HIF1α and HIF2α activity.

HIF and Kidney Cancer

In pVHL-defective ccRCC, polyubiquitylation of HIFα is impaired, leading to inappropriate activation of HIF target genes under normoxic conditions. HIF activates a number of genes that are believed to play, or suspected of playing, roles in kidney carcinogenesis, including TGFα, Cyclin D1, SDF1, CXCR4, MMP family members, PDGF B, and VEGF. The increased expression of factors such as VEGF likely contributes to the highly angiogenic nature of clear cell renal carcinomas and their responsiveness to VEGF blockade. The overproduction of the canonical HIF target erythropoietin accounts for the association of VHL-associated neoplasms, including renal carcinomas, with paraneoplastic

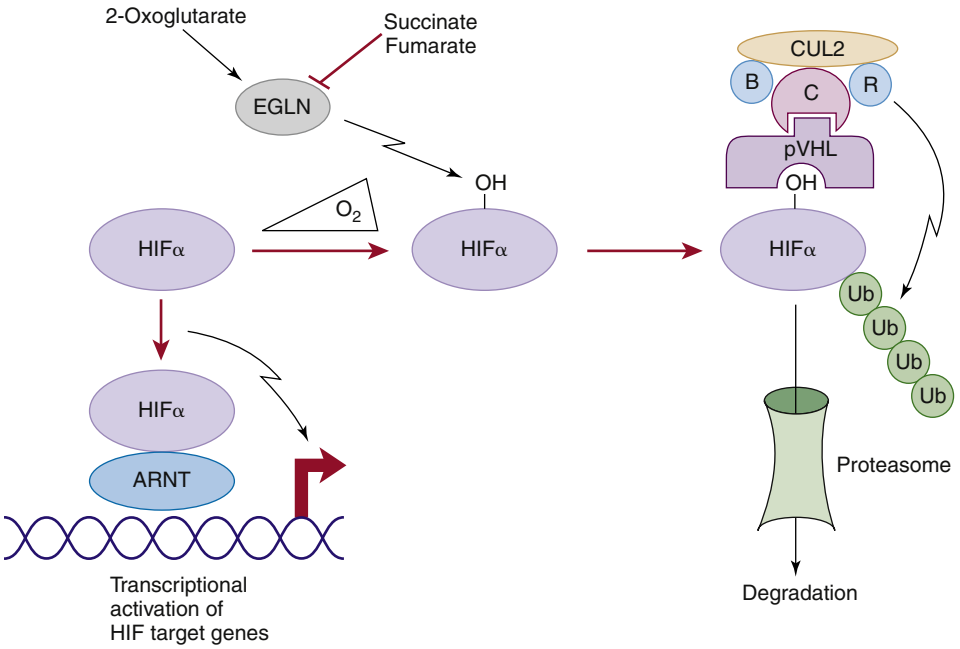


Figure 39-1 Regulation of HIF by pVHL In the presence of oxygen, HIFα is hydroxylated on one (or both) of two conserved prolyl residues by EglN family members. This creates a binding site for pVHL, which is stably bound to elongin B (*B*), elongin C (*C*), Cul2, and Rbx1 (*R*). This complex then polyubiquitylates HIFα, marking it for proteasomal degradation. Under low-oxygen conditions, or in cells lacking pVHL, HIFα binds to ARNT, translocates to the nucleus, and activates HIF target genes such as *VEGF*. EglN family members require 2-oxoglutarate, in addition to oxygen, and are inhibited by succinate and fumarate.

erythrocytosis.[15] HIFα, acting without its partner HIFβ, can also activate Notch signaling, which has been implicated in renal carcinogenesis.

Several lines of evidence suggest that deregulation of HIF, and particularly deregulation of HIF2α, plays a causal role in pVHL-defective renal carcinoma. To date virtually all kidney cancer–associated *VHL* mutations, whether germline or somatic, compromise pVHL's ability to regulate HIF, and the risk of developing kidney cancer in VHL families correlates well with the degree to which their mutant *VHL* alleles deregulate HIF. Close examination of preneoplastic renal lesions in VHL patients reveals that the appearance of HIF2α is associated with histological changes indicative of incipient malignant transformation.

In stark contrast to HIF2α, HIF1α appears to act as a renal cancer suppressor. *HIF1α* resides on chromosome 14q, which is frequently deleted in kidney cancers. *HIF1α* mRNA and protein is decreased in a sizable fraction of clear cell renal tumors, and HIF1α-responsive mRNAs are diminished in 14q-deleted renal tumors. It should be noted, however, that most 14q-deleted clear cell renal tumors, in contrast to cell lines, appear to retain a wild-type *HIF1α* allele. It is therefore possible that haploinsufficiency of *HIF1α* promotes tumor growth in vivo and that reduction to nullizygosity occurs during tumor progression (most cancer lines are derived from metastatic lesions) or establishment and propagation of cell lines. Although rare, intragenic *HIF1α* mutations have been reported in kidney cancers. Collectively, these findings suggest that *HIF1α* is a target of the 14q deletions that are typical of clear cell renal carcinomas.

The differential effects of HIF1α and HIF2α in pVHL-defective tumor growth likely reflect both qualitative and quantitative differences. For example, the genes regulated by HIF1α and HIF2α overlap but are clearly not identical. Perhaps HIF1α preferentially induces the expression of a renal tumor suppressor, or suppressors, whereas HIF2α preferentially induces the expression of one or more downstream oncogenes. Moreover, HIF1α can potentially antagonize HIF2α with respect to the induction of shared targets under normoxic conditions in pVHL-defective cells because transcriptional activation by HIF1α, in contrast to HIF2α, is enfeebled by the oxygen-dependent asparaginyl hydroxylase FIH1.

HIF-Independent pVHL Functions

pVHL has a number of other functions that appear to be at least partially HIF-independent and that might contribute to renal cancer suppression. For example, pVHL promotes fibronectin matrix assembly, maintains microtubule stability and a specialized structure called the primary cilium, suppresses the activating phosphorylation of the NFκB agonist CARD9, suppresses signaling by HGF and c-MET, suppresses Wnt signaling, inhibits atypical PKC activity, promotes the Cbl-independent ubiquitylation of EGFR, and regulates senescence. The ciliary defect in pVHL-defective renal epithelial cells is particularly intriguing because renal cysts are a prominent feature of both VHL disease as well as the other primary ciliopathies.

Mouse Models of VHL Disease

There are currently no genetically engineered mouse models that develop *VHL−/−* clear cell renal carcinomas. *VHL−/−* embryos

die before term, and *VHL*+/– do not develop the usual stigmata of VHL disease. They do, however, develop hepatic blood vessel lesions that are associated with stochastic loss of the remaining wild-type *VHL* allele and HIF activation. *VHL* has also been inactivated using conditional alleles in a variety of mouse tissues, leading to phenotypes such as renal cysts (kidney), hepatosteatosis and hemangiomas (liver), cardiomyopathy (heart), and increased angiogenesis and weight loss (skin). Concurrent inactivation of *PTEN* in the kidney exacerbates the renal cyst phenotype but still does not lead to renal tumor formation. In most of the models tested so far, deregulation of HIF2α appears to be necessary and sufficient for the pathology observed on after pVHL loss in mouse tissues.

Other Hereditary Forms of Cancer

Individuals with germline activating *c-Met* mutations are highly predisposed to Type 1 papillary renal carcinoma, often associated with duplication of the mutant *c-Met* allele located at 7q31[16,17] (see Table 39-1). *c-Met* encodes a receptor tyrosine kinase that influences cellular proliferation, survival, invasion, and metastasis. Somatic *c-Met* mutations are relatively rare in sporadic papillary renal carcinomas (see Table 39-3).

Germline loss-of-function fumarate hydratase (1q42) mutations cause a syndrome characterized by Type 2 papillary renal carcinoma, cutaneous leiomyomata, and uterine fibroids, whereas succinate dehydrogenase subunit mutations cause familial paragangliomas and, rarely, clear cell renal carcinomas (see Table 39-1). Inactivation of fumarate hydratase and succinate dehydrogenase leads to the intracellular accumulation of fumarate and succinate, respectively, which competitively inhibit 2-oxoglutarate-dependent enzymes including the EglN prolyl hydroxylases (see Figure 39-1). Accordingly, HIFα levels are increased in *FH*–/– and *SDH*–/– tumors. It has also been shown that the secondary accumulation of succinate in fumarate hydratase–deficient cells can also, through covalent linkage to a cysteine residue, inactivate the KEAP tumor suppressor protein, leading to deregulation of the NRF2 transcription factor.

Inactivating germline mutations of *FLCN* (17p11.2) cause Birt-Hogg-Dube syndrome, which is characterized by a variety of dermatological lesions, including fibrofolliculomas; renal tumors including oncocytomas and chromophobe tumors and, less commonly, papillary and clear cell carcinomas; and pulmonary cysts (see Table 39-1). The *FLCN* gene product, Folliculin, binds to FNIP1 and FNIP2 and is believed to modulate nutrient sensing by the mTOR signaling pathway. Folliculin also appears to suppress TGFβ

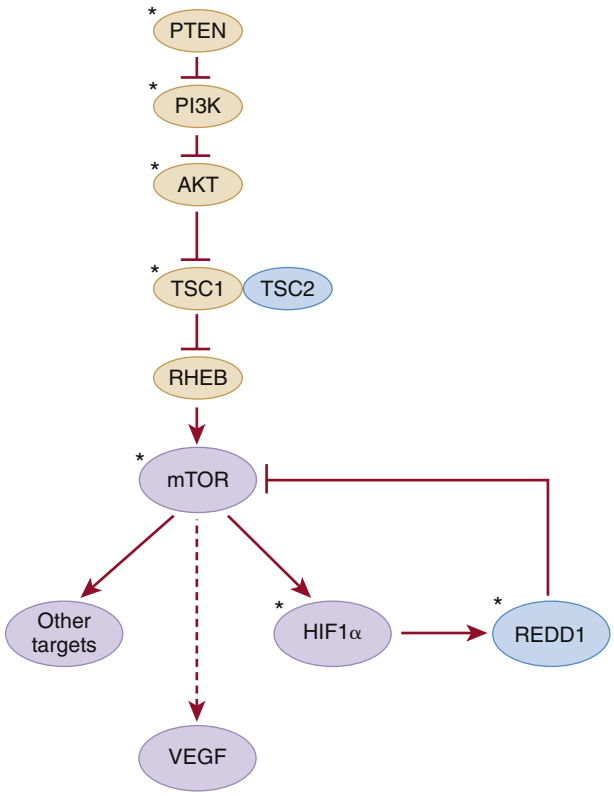


Figure 39-2 Kidney cancer mutations with an impact on mTOR Proteins that have been targets of mutations in kidney cancer are indicated by *asterisks*.

as well as the expression of TFE3, the common partner in kidney cancers linked to Xp11.2 translocations. Inactivating mutations of either *TSC1* (9q34), which encodes hamartin, or *TSC2* (16p13), which encodes tuberin, cause tuberous sclerosis. Tuberous sclerosis is associated with cutaneous, neurological, and renal abnormalities. Renal abnormalities include angiomyolipomata and, less commonly, clear cell renal carcinoma (see Table 39-1). Hamartin and tuberin form a complex that inhibits the mTOR kinase and regulates VEGF via both mTOR-dependent and independent pathways (Figure 39-2). Somatic *TSC1* mutations have been described in sporadic ccRCC, as have mutations in other genes whose products link growth factor signaling, via hamartin and tuberin, to mTOR activity (see later discussion). Inactivation of the hamartin/tuberin complex in mice causes renal cysts, and a germline *TSC2* mutation in the rat (Eker Rat) causes clear cell carcinoma.

Some families predisposed to clear cell carcinoma carry germline translocations involving chromosome 3 and variable partners (see Table 39-1). Loss of the derivative chromosome 3p segment is believed to cause loss of tumor suppressors, such as *VHL*, located between 3p21 and 3p25.

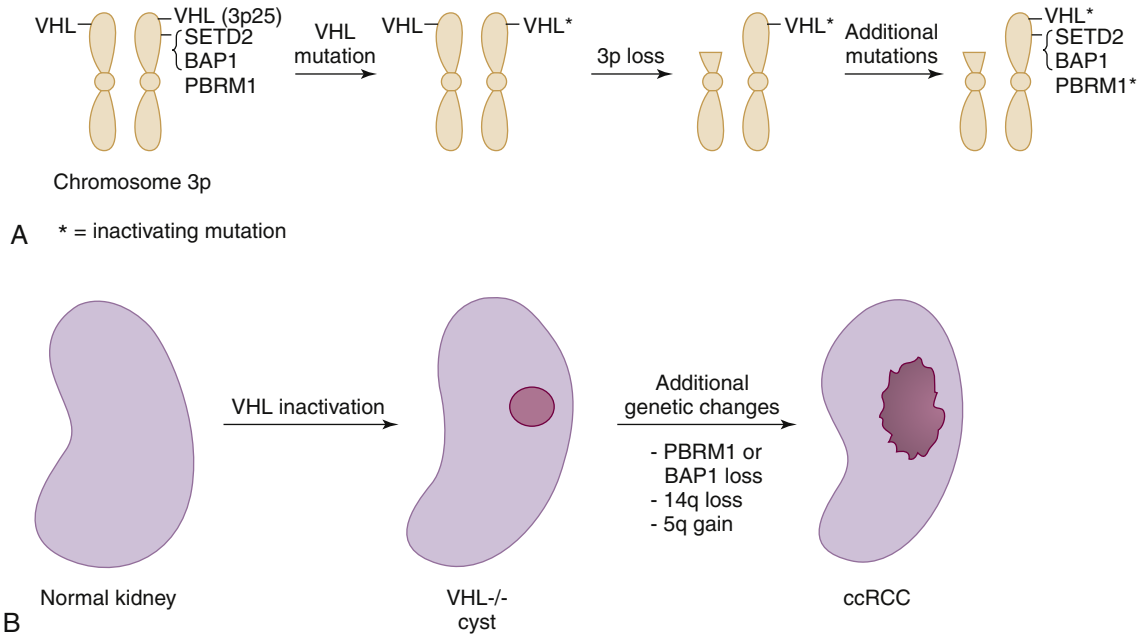


Figure 39-3 Multistep kidney carcinogenesis (A) Multiple renal cancer suppressors are located on chromosome 3p, including *VHL* at 3p25 and *SETD2*, *BAP1*, and *PBRM1* on 3p21. For this reason three genetic "hits" can inactivate two tumor suppressors (and four "hits," three tumor suppressors) provided one of the hits is chromosome 3p loss. In the example shown both *VHL* and *PBRM1* have been inactivated. **(B)** pVHL loss leads to preneoplastic renal lesions, such as *VHL–/–* renal cysts. In VHL disease the kidney is germline *VHL+/–*, whereas in sporadic kidney cancers the kidney is germline *VHL+/+*. Additional genetic changes, such as *PBRM1* or *BAP1* mutations, loss of chromosome 14q, and gain of chromosome 5q, are linked to progression to clear cell renal carcinoma.

Common Somatic Alterations in Clear Cell Renal Carcinoma

Copy Number Changes

The most common copy number changes in clear cell renal carcinoma relate to loss of chromosome 3p, which is observed in more than 90% of these tumors (see Table 39-3). Chromosome 3p harbors several tumor suppressor genes, including the *VHL* gene at 3p25, and *PBRM1*, *BAP1*, and *SETD2* at 3p21 (Figure 39-3, *A*). Other common copy number changes in clear cell renal carcinoma include amplification of chromosome 5q and 7q and loss of chromosome 14q and 6q. Loss of chromosome 3p, gain of 5q, and loss of 14q are seen more commonly in clear cell renal carcinoma than in other cancers.

Inactivation of Tumor Suppressor Genes

PBRM1

PBRM1, located on 3p21 (see Figure 39-3, *A*), is mutationally inactivated in about 40% of ccRCC (see Table 39-3), typically as a result of truncating mutations of one allele coupled with loss of the remaining allele as a consequence of chromosome 3p loss. *PBRM1* mutations are not mutually exclusive with mutations of the neighboring gene *SETD2*, indicating that the two genes are not wholly redundant. *PBRM1* encodes a large protein called BAF180, which contains six bromodomains, 2 bromo-adjacent homology domains, and an HMG DNA-binding domain. BAF180 is a component of the PBAF SWI/SNF nucleosome remodeling complex and is therefore likely to affect transcription by altering chromatin structure so as to alter its accessibility to various transcription factors (Figure 39-4, *A*).

BAP1

BAP1, which is also located on chromosome 3p21 (see Figure 39-3, *A*), is mutationally inactivated in about 15% of clear cell renal carcinomas (see Table 39-3). As is true for *PBRM1*, *BAP1* mutations in kidney cancer are typically truncating mutations that are presumed to be loss of function. Loss-of-function *BAP1* mutations, including germline mutations, have also been linked to other tumors. Interestingly, families with germline *BAP1* mutations may also be predisposed to develop clear cell renal carcinoma. BAP1, which was originally identified as a BRCA1-associated protein, encodes a nuclear deubiquitinase of the C-terminal hydrolase (UCH)-domain family. Suppression of renal proliferation by BAP1 apparently does not require its ability to deubiquitinate its canonical target, histone H2A, but does require that it bind

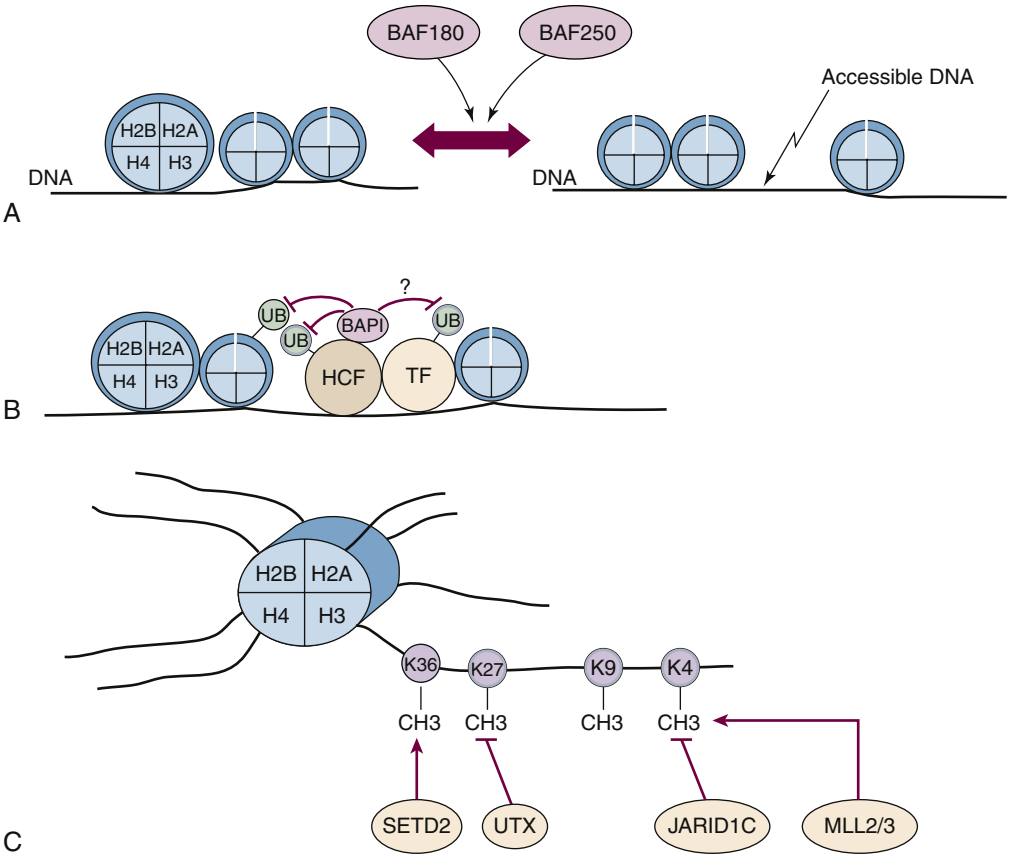


Figure 39-4 Kidney cancer mutations with an impact on chromatin regulation **(A)** The *PBRM1* gene product BAF180 and *ARID1A* gene product BAF250 are components of SWI/SNF complexes that can reposition nucleosomes. **(B)** BAP1 binds to HCF and can potentially deubiquitinate histones, adjacent transcription factors (TF), and HCF itself. **(C)** SETD2, MLL2, and MLL3 methylate specific lysine residues in histone H3, whereas UTX and JARID1 demethylate specific lysine residues. Note that for simplicity, the other components of multiprotein complexes such as the SWI/SNF complexes and MLL complexes are not shown.

to host cell factor-1 (HCF) (see Figure 39-4, *B*). HCF is a transcriptional coactivator that interacts with a number of transcription factors, including E2F family members and histone-modifying enzyme complexes, including histone methyltransferases and acetyltransferases.

SETD2

SETD2, like *PBRM1* and *BAP1*, is located on chromosome 3p21 (see Figure 39-3, *A*). It is mutationally inactivated in about 5% of clear cell renal carcinomas. Loss-of-function *SETD2* mutations have also been found in breast cancers. *SETD2* encodes a SET-domain–containing histone methyltransferase that promotes H3K36 methylation (see Figure 39-4, *C*). H3K36 trimethylation is undetectable in cells lacking wild-type SETD2, suggesting that SETD2 is not redundant with other histone methyltransferases.

UTX/KDM6A

UTX/KDM6A, located on the X chromosome but escaping X inactivation, is mutated in about 2% of clear cell renal carcinomas. *UTX* loss-of-function mutations have been described in a variety of cancers, including hematological malignancies and prostate cancer. *UTX/KDM6A* encodes an H3K27 demethylase (see Figure 39-4, *C*). Interestingly, *EZH2*, which encodes an H3K27 methylase, is overexpressed in renal cancer and promotes renal carcinoma proliferation and survival in cell culture experiments. Moreover, UTX can bind to H3K4 demethylase complexes that contain MLL2 or MLL3, which are mutationally inactivated in a small subset of kidney cancers. Loss of UTX leads to silencing of genes required for pRB-dependent cell-cycle arrest and also leads to activation of the Notch pathway, which has recently been implicated in renal carcinogenesis.

JARID1C

JARID1C/KDM5C, which is also located on the X chromosome as an actively transcribed unit, is mutated in about 3% of clear cell renal carcinomas. JARID1C is an H3K4 demethylase (see Figure 39-4, C). As is true for many other histone demethylases, including JMJD1A and JMJD2B, JARID1C is induced by HIF and hence potentially deregulated in pVHL-defective tumor cells. Increased expression of JARID1C in pVHL-defective tumor cells decreases H3K4 methylation and decreases cell proliferation in vitro and in vivo.

MLL2 and MLL3

MLL2 (12q13) and *MLL3* (7q36) are both mutated at low frequency (1% to 3% each) in clear cell renal carcinoma. As indicated earlier, these genes encode proteins that participate in multiprotein complexes that promote H3K4 methylation and that interact with the UTX tumor suppressor (see Figure 39-4, *C*).

mTOR

Activating mutations of the mTOR kinase gene (1p36) have been described in ccRCC. mTOR plays a critical role in transducing signals related to nutrient and energy availability. Mutations affecting individual components of the mTOR pathway, including mTOR itself, are relatively uncommon in ccRCC but, when viewed in aggregate, support that activation of the mTOR pathway promotes clear cell renal carcinogenesis (see Figure 39-2). Moreover, as described earlier, germline inactivation of the mTOR suppressors hamartin and tuberin has been linked to ccRCC in rodents and people.

Other Genes

Other genes mutated at a low frequency (less than 3%) in clear cell renal carcinoma include the cancer-relevant genes *PTEN*, *PIK3CA*, *AKT1*, *TP53*, *ATM*, *TSC1*, *REDD1*, and *ARID1A*. PTEN, PI3KCA, AKT1, and TSC1 participate in the mTOR pathway (see Figure 39-2). *TP53* mutations, when they occur, have been linked to sarcomatoid changes in ccRCC. *ARID1A* encodes BAF250, which, like BAF180, functions in a chromatin remodeling complex (see Figure 39-4, *A*).

Multistep Genetic Models of Clear Cell Renal Carcinoma

The remaining wild-type *VHL* allele is lost in the numerous preneoplastic renal cysts that develop in patients with von Hippel-Lindau disease.[18] This suggests that additional genetic alterations involving other loci are required to convert these lesions to clear cell renal carcinomas. These alterations presumably involve the copy number changes (such as chromosome 5q amplification and chromosome 14q loss) and intragenic mutations described earlier (such as *PBRM1* or *BAP1* mutations) (see Figure 39-4, *B*). Conceivably one or more of these changes involves circumventing the antiproliferative effects of HIF1α, which in model systems can be accomplished by downregulation of HIF1α itself or by activation of the mTOR pathway. Other changes—for example, mutational inactivation of chromatin modifiers such as BAF180 or JARD1C—might compensate for HIF-driven changes in chromatin structure.

PBRM1 and *BAP1* mutations appear to be largely mutually exclusive, suggesting they affect a common pathway. Arguing against this, however, are the observations that *PBRM1* and *BAP1* mutant tumors have very distinctive transcriptional and signal transduction profiles. It should be noted that kidney cancers do rarely mutate both of these genes, but in these cases the tumors assume rhabdoid features. Deep sequencing of multiple spatially distinct biopsy specimens has recently been used to examine intratumoral heterogeneity within clear cell carcinomas. These studies confirm that clear cell carcinomas evolve over time and can contain multiple subclones that are heterogeneous with respect to their genetic changes subsequent to *VHL* loss.

Common Somatic Alterations in Non–Clear Cell Renal Carcinoma

In contrast to the *VHL* gene, the hereditary kidney cancer genes *c-MET* and *FH* are rarely mutated in sporadic kidney cancers. Somatic *FLCN* mutations have been identified in sporadic chromophobe tumors. Somatic Xp11.2 translocations should be suspected in kidney cancers arising in children and young adults. The usual *TFE3* translocation partners are *ASPL*, *PSF*, *NONO*, *CLTC*, or *PRCC*. Although the mechanism(s) through which Xp11.2 translocations drive kidney tumorigenesis remain largely unknown, it has been recently shown that TFE3 fusion proteins transactivate the *c-MET* promoter and induce autophosphorylation of the MET protein and activation of downstream signaling. Inhibition of MET signaling in cell lines containing endogenous TFE3 fusion proteins causes a decrease in cell growth.

In general, non–clear cell renal carcinomas, because of their rarity relative to clear cell carcinomas, have been less intensively surveyed with respect to intragenic mutations. Each histological type of sporadic kidney cancer does, however, display stereotypical copy number alterations[2] (see Table 39-3). For example, trisomy of chromosome 7 and/or 17 is typical of papillary renal carcinomas, and loss of chromosome 1, 14, and 15 is typical of mucinous tubular and spindle carcinomas. Chromophobe tumors are characterized by loss of genomic material from chromosomes 1, 2, 6, 10, 13, 17, and/or 21.

Clinical Applications of Molecular Insights

Early Detection and Risk Assessment

Kidney cancer can be cured by surgery when it is localized. Therefore screening could, in theory, lower kidney cancer mortality. A major problem for kidney cancer screening, however, is the overall low prevalence of kidney cancer. One study of abdominal ultrasonographic (US) screening of 219,640 persons confirmed RCC in 192 cases (0.09%), and another US study of 60,604 persons found an RCC prevalence of 0.02%. Screening based on computed tomography (CT) has revealed a prevalence of asymptomatic kidney cancer of approximately 0.2%.[19] Moreover, screening for RCC based on anatomical imaging has a high false-positive rate. In one study, 37% of patients with renal tumors smaller than 1 cm detected by screening had benign lesions based on pathological review. Screening with US or CT may be useful in high-risk populations such as patients on dialysis or those with established hereditary kidney cancer syndromes such as VHL disease. In patients with VHL disease, screening for RCC is very important to detect new lesions at an early stage and to monitor the growth of small asymptomatic lesions. There is no standard protocol for screening or surveillance for such patients, but several recommendations exist, such as those adopted by the VHL Family Alliance (www.vhl.org). The treatment approach to kidney cancer in patients with VHL syndrome has shifted from radical nephrectomy to nephron-sparing approaches (surveillance, partial nephrectomy, and thermal ablation). Small tumors are generally observed, as they are at very low risk for metastatic progression as illustrated in a series of 108 patients from the National Cancer Institute, where no metastases were detected in patients with tumors less than 3 cm in diameter.[20]

Therapy (Clear Cell)

Conventional chemotherapy has little role in the management of metastatic kidney cancer. High-dose interleukin 2 remains the only treatment with a small (less than 10%) but measurable probability of inducing durable remissions in this patient population.

VEGF

The knowledge that ccRCC is usually driven by deregulation of HIF, and HIF targets genes such as VEGF, provided a conceptual foundation for testing VEGF antagonists for the treatment of metastatic kidney cancer. In clinical trials about 70% of kidney cancer patients will achieve some degree of tumor shrinkage when treated with such agents. In randomized trials this is associated with an improvement in progression-free survival. Based on such trials, five drugs that either neutralize VEGF or block VEGFR have now been approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic kidney cancer (Table 39-4). VEGF inhibitors, although clearly active

Table 39-4 Landmark Phase III Trials of Approved Targeted Agents in Metastatic RCC

Trial	N	Setting	Risk Group(s)	PFS (months)	ORR (%)	OS (months)
Sunitinib vs. IFN-α	750	Treatment naive	Good/intermediate	11 vs. 5	47 vs. 12	26.4 vs. 21.8
Pazopanib vs. BSC	435	Treatment naive or prior cytokines	Good/intermediate	9.2 vs. 4.2 (all) 11.1 vs. 2.8 (untreated)	30 vs. 3	22.9 vs. 20.5
Bevacizumab + IFN-α vs. IFN-α/placebo	649	Treatment naive	Good/intermediate	10.2 vs. 5.4	31 vs. 13	23.3 vs. 21.3
Bevacizumab + IFN-α vs. IFN-α	732	Treatment naive	Good/intermediate	8.5 vs. 5.2	26 vs. 13	18.3 vs. 17.4
Temsirolimus vs. IFN-α	626	Treatment naive	Poor	3.8 vs. 1.9	9 vs. 5	10.9 vs. 7.3
Sorafenib vs. placebo	903	Treatment naive or prior cytokines	Good/intermediate	5.5 vs. 2.8	11 vs. 2	17.8 vs. 15.2
Everolimus + BSC vs. placebo + BSC	416	Prior sorafenib or sunitinib	All risk groups	4.9 vs. 1.9	2 vs. 0	14.8 vs. 14.4
Axitinib vs. sorafenib	723	One prior therapy	All risk groups	6.7 vs. 4.7 4.8 vs. 3.4 (sunitinib-pretreated) 12.1 vs. 6.5 (cytokine-pretreated)	19 vs. 9	Not mature

BSC, Best supportive care; *ORR*, overall response rate; *OS*, overall survival; *PFS*, progression-free survival.

in kidney cancer, are not curative. Newer, more potent VEGF inhibitors are currently being tested, although there is already an indication that VEGF blockade above a certain threshold will begin to cause on-target side effects such as microangiopathic anemia, cardiomyopathy, and severe hypertension.

mTOR

mTOR regulates both the transcription and translation of HIF and also acts downstream of VEGF in endothelial cells. In addition, mutations affecting the mTOR pathway (*PTEN*, *PIK3CA*, and *mTOR*), although rare, have been documented in ccRCC. Finally, pVHL-defective cells display an increased requirement for mTOR relative to pVHL-proficient cells. Two rapamycin-like mTOR inhibitors (rapalogs), temsirolimus and everolimus, have now also been approved for the treatment of kidney cancer based on favorable randomized trial data (see Table 39-4). As is true for VEGF inhibitors, temsirolimus and everolimus are not curative treatments for kidney cancer. Moreover, rapalogs such as these preferentially inhibit mTOR when it exists in the TORC1 complex relative to the TORC2 complex. This is potentially important because downregulation of HIF2α, which is believed to drive pVHL-defective ccRCC, requires that TORC2 be suppressed. Preclinical data suggest that small molecules that block both TORC1 and TORC2 will be more active than rapalogs for the treatment of kidney cancer.[21]

EGFR

pVHL loss, as described earlier, upregulates EGFR as well as its ligand, TGFα. Moreover, blocking EGFR activity inhibits pVHL-defective ccRCC tumor growth in preclinical models. To date, however, EGFR inhibitors have been disappointing for the treatment of human kidney cancer. This could reflect a failure to achieve sufficient EGFR inhibition or the fact that MET, which becomes activated on pVHL loss, provides collateral survival signals that render cells resistant to EGFR inhibitors, as has been described in other settings.[22]

Therapy (Non–Clear Cell)

Both VEGF inhibitors and mTOR inhibitors have some activity in non-clear cell RCC. Clinical studies with MET inhibitors for pRCC and rapamycin-like drugs for chRCC are anticipated. Rapalogs also appear to be modestly active in the treatment of renal angiomyolipomata.

Prognostic and Predictive Markers

Renal cell carcinoma remains a disease with widely varying clinical outcome. In nonmetastatic RCC, routine prognostic factors are TNM stage, Fuhrman grade, and symptoms at presentation. Gene expression profiling and certain genetic polymorphisms represent some of the promising prognostic biomarkers in localized RCC[23] and may add to established clinical factors.

In metastatic RCC, several host- and tumor-related factors have been identified as important prognostic factors and have been integrated into prognostic models. Of these the most commonly used system is the Memorial Sloan Kettering Cancer Center model. This model stratifies patients with metastatic RCC into poor-, intermediate-, and favorable-risk categories (with distinct survival patterns) based on the number of adverse clinical (performance status, time from diagnosis to systemic therapy) and laboratory (serum hemoglobin, corrected calcium and lactate dehydrogenase [LDH]) parameters present. Unfortunately, there are currently no predictive biomarkers that can help in the selection of systemic therapy in metastatic disease, and most data are retrospective in nature. Single-nucleotide polymorphisms (SNPs) in genes involved in angiogenesis, such as *VEGF* and *IL-8*, or involved in VEGFR inhibitor drug metabolism, may be associated with clinical outcome and with selected toxicities. For mTOR inhibitors, it remains to be determined whether mTOR mutations or mutations affecting the mTOR pathway are predictive biomarkers in this setting. One major challenge for developing personalized therapy in RCC is intratumor heterogeneity manifested by different regions of the same tumor bearing different mutations and having very distinct gene expression profiles.

Summary and Future Directions

Most kidney cancers are renal cell carcinomas, and most renal cell carcinomas are clear cell renal carcinomas. Inactivation of the *VHL* tumor suppressor gene is an early gatekeeper event in clear cell renal carcinoma and leads to deregulation of the HIF transcription factor and HIF target genes. HIF2α acts as a kidney cancer oncoprotein, whereas HIF1α appears to serve as a kidney cancer tumor suppressor. Cooperating mutations in pVHL-defective clear cell renal carcinomas include loss of chromosome 14q, gain

of chromosome 5q, and intragenic mutations affecting a number of genes involved in chromatin regulation, such as *PBRM1* and *BAP1*. The genetic alterations responsible for the other major forms of kidney cancers are beginning to come into view, aided by studies of rare families that are predisposed to specific forms of RCC.

Treatment of metastatic kidney cancer has historically been difficult and restricted largely to immunomodulators. Knowledge of the VHL-HIF-VEGF pathway provided a conceptual foundation for the successful testing of VEGF and mTOR inhibitors for the treatment of clear cell carcinoma. These drugs are not curative, however, as single agents. Deeper understanding of kidney cancer pathogenesis will, it is hoped, yield additional targets that, when inhibited, synergize with existing therapies such as VEGF blockade.

Key References

1. Chow WH, Dong LM, Devesa SS. Epidemiology and risk factors for kidney cancer. *Nat Rev Urol*; 7:245–257.
2. Linehan WM, Pinto PA, Bratslavsky G, et al. Hereditary kidney cancer: unique opportunity for disease-based therapy. *Cancer*. 2009;115:2252-2261.
3. Purdue MP, Johansson M, Zelenika D, et al. Genome-wide association study of renal cell carcinoma identifies two susceptibility loci on 2p21 and 11q13.3. *Nat Genet*. 2011;43:60-65.
4. Schodel J, Bardella C, Sciesielski LK, et al. Common genetic variants at the 11q13.3 renal cancer susceptibility locus influence binding of HIF to an enhancer of cyclin D1 expression. *Nat Genet*. 2012;44:420-425 S1-S2.
5. Leibovich BC, Lohse CM, Crispen PL, et al. Histological subtype is an independent predictor of outcome for patients with renal cell carcinoma. *J Urol*; 183:1309-1315.
6. Linehan WM. The genetic basis of kidney cancer: implications for management and use of targeted therapeutic approaches. *Eur Urol*; 61:896–898.
7. Maher E, Kaelin WG. von Hippel-Lindau Disease. *Medicine*. 1997;76:381-391.
8. Iliopoulos O, Ohh M, Kaelin W. pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A*. 1998;95:11661-11666.
9. Blankenship C, Naglich J, Whaley J, Seizinger B, Kley N. Alternate choice of initiation codon produces a biologically active product of the von Hippel Lindau gene with tumor suppressor activity. *Oncogene*. 1999;18:1529-1535.
10. Schoenfeld A, Davidowitz E, Burk R. A second major native von Hippel-Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. *Proc Natl Acad Sci U S A 1*. 1998;95:8817-8822.
11. Kaelin WG. Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer*. 2002;2:673-682.
12. Semenza GL. Oxygen sensing, homeostasis, and disease. *N Engl J Med*. 2011;365:537-547.
13. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399-408.
14. Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30:393-402.
15. Golde DW, Hocking WG. Polycythemia: mechanisms and management. *Ann Intern Med*. 1981;95:71-87.
16. Linehan WM, Bratslavsky G, Pinto PA, et al. Molecular diagnosis and therapy of kidney cancer. *Annu Rev Med*; 61:329-343.
17. Schmidt L, Duh F, Chen F, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nature Genetics*. 1997;16:68-73.
18. Mandriota SJ, Turner KJ, Davies DR, et al. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer Cell*. 2002;1:459-468.
19. Fenton JJ, Weiss NS. Screening computed tomography: will it result in overdiagnosis of renal carcinoma? *Cancer*. 2004;100:986-990.
20. Duffey BG, Choyke PL, Glenn G, et al. The relationship between renal tumor size and metastases in patients with von Hippel-Lindau disease. *J Urol*. 2004;172:63-65.
21. Cho DC, Cohen MB, Panka DJ, et al. The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. *Clin Cancer Res*. 2010;16:3628-3638.
22. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007;316:1039-1043.
23. Schutz FA, Gray KP, Pomerantz M, et al. Do genetic polymorphisms predict risk of recurrence in patients with localized renal cell carcinoma? Results from a cohort study. *J Clin Oncol*. 2011;29 suppl; abstr 4506.

This page intentionally left blank

References

1. Chow WH, Dong LM, Devesa SS. Epidemiology and risk factors for kidney cancer. *Nat Rev Urol*; 7:245–257.
2. Linehan WM, Pinto PA, Bratslavsky G, et al. Hereditary kidney cancer: unique opportunity for disease-based therapy. *Cancer*. 2009;115:2252-2261.
3. Purdue MP, Johansson M, Zelenika D, et al. Genome-wide association study of renal cell carcinoma identifies two susceptibility loci on 2p21 and 11q13.3. *Nat Genet*. 2011;43:60-65.
4. Schodel J, Bardella C, Sciesielski LK, et al. Common genetic variants at the 11q13.3 renal cancer susceptibility locus influence binding of HIF to an enhancer of cyclin D1 expression. *Nat Genet*. 2012;44:420-425 S1-S2.
5. Leibovich BC, Lohse CM, Crispen PL, et al. Histological subtype is an independent predictor of outcome for patients with renal cell carcinoma. *J Urol*; 183:1309-1315.
6. Linehan WM. The genetic basis of kidney cancer: implications for management and use of targeted therapeutic approaches. *Eur Urol*; 61:896-898.
7. Maher E, Kaelin WG. von Hippel-Lindau Disease. *Medicine*. 1997;76:381-391.
8. Iliopoulos O, Ohh M, Kaelin W. pVHL19 is a biologically active product of the von Hippel-Lindau gene arising from internal translation initiation. *Proc Natl Acad Sci U S A*. 1998;95:11661-11666.
9. Blankenship C, Naglich J, Whaley J, Seizinger B, Kley N. Alternate choice of initiation codon produces a biologically active product of the von Hippel Lindau gene with tumor suppressor activity. *Oncogene*. 1999;18:1529-1535.
10. Schoenfeld A, Davidowitz E, Burk R. A second major native von Hippel-Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. *Proc Natl Acad Sci U S A 1*. 1998;95:8817-8822.
11. Kaelin WG. Molecular basis of the VHL hereditary cancer syndrome. *Nat Rev Cancer*. 2002;2:673-682.
12. Semenza GL. Oxygen sensing, homeostasis, and disease. *N Engl J Med*. 2011;365:537-547.
13. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399-408.
14. Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. 2008;30:393-402.
15. Golde DW, Hocking WG. Polycythemia: mechanisms and management. *Ann Intern Med*. 1981;95:71-87.
16. Linehan WM, Bratslavsky G, Pinto PA, et al. Molecular diagnosis and therapy of kidney cancer. *Annu Rev Med*; 61:329-343.
17. Schmidt L, Duh F, Chen F, et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nature Genetics*. 1997;16:68-73.
18. Mandriota SJ, Turner KJ, Davies DR, et al. HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron. *Cancer Cell*. 2002;1:459-468.
19. Fenton JJ, Weiss NS. Screening computed tomography: will it result in overdiagnosis of renal carcinoma? *Cancer*. 2004;100:986-990.
20. Duffey BG, Choyke PL, Glenn G, et al. The relationship between renal tumor size and metastases in patients with von Hippel-Lindau disease. *J Urol*. 2004;172:63-65.
21. Cho DC, Cohen MB, Panka DJ, et al. The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. *Clin Cancer Res*. 2010;16:3628-3638.
22. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007;316:1039-1043.
23. Schutz FA, Gray KP, Pomerantz M, et al. Do genetic polymorphisms predict risk of recurrence in patients with localized renal cell carcinoma? Results from a cohort study. *J Clin Oncol*. 2011;29 suppl; abstr 4506.

Matthew C. Havrda and Mark A. Israel

40

The Biology of Primary Brain Tumors

Malignant primary tumors of the central nervous system (CNS) occur in about 25,500 individuals and account for an estimated 13,700 deaths in the United States annually, a mortality rate of 6.5 per 100,000. Based on most recent reports, benign tumors of the CNS are about twice as common as malignant brain tumors, but with a much lower mortality rate.[1,2] Overall, CNS cancer is estimated to represent about 1% of newly occurring malignant tumors. In children and young adults, brain tumors are a major health problem second only to leukemia as a cause of cancer-related deaths, and they are the third leading cause of cancer-related death between ages 15 and 34. The age-adjusted incidence appears to be similar to that observed in other developed countries.

Brain tumors are a diverse group of neoplasms. The particular combination of somatic genetic alterations found in different brain tumors that are histologically distinguishable can vary considerably among individual examples of a particular tumor type. Similarly, extensive molecular and genetic variation is well documented within tumors that are histologically indistinguishable.[3] Recognition of a number of hereditary and nonfamilial syndromes in which brain tumors play a prominent role or occur with increased frequency provide compelling evidence for the importance of several cell regulatory pathways in brain tumor pathogenesis (Table 40-1). Primary CNS tumors have been classified by surgeons and pathologists on the basis of their location and histologic appearance, providing important information that guides treatment with conventional antineoplastic modalities including surgery, irradiation, and chemotherapy as well as contributing to prognostication. More recently, the drive toward more targeted therapy has focused attention on approaches to classification emphasizing tumor type–specific molecular alterations. Glial tumors account for 50% to 60% of all primary brain tumors, and with the exception of some pilocytic astrocytoma, most are malignant. Astrocytomas account for the great majority of the glial tumors, whereas the second most common type is oligodendroglioma. Exposure to ionizing radiation is the only well-documented environmental risk factor for the development of glioma. Benign CNS tumors consist primarily of meningioma and low-grade glial tumors.

Hereditary Syndromes and Central Nervous System Oncogenesis

In addition to rare families in which there is strong evidence for a hereditary basis for the development of familial meningioma or glioma, a number of well-studied cancer predisposition syndromes include among their associated stigmata the development of CNS tumors (see Table 40-1). These are important clinical observations and have provided insights into the pathogenesis of several different types of primary brain tumors. Typically these syndromes are associated not only with an increased incidence of brain tumors, but also with the occurrence of tumors at an earlier age than those arising spontaneously and with the finding of multicentric tumors. Inherited syndromes associated with brain tumors are inherited as autosomal dominant disorders and arise as the result of the germline mutation of one allele of a tumor suppressor gene whose other copy is typically inactivated in the tumors that arise. The most commonly occurring brain tumor predisposition syndrome is neurofibromatosis type 1 (NF1).[4] NF1 affects approximately 1 in 3000 live births; approximately 15% of these have radiographic evidence of optic glioma early in childhood, although most cases do not become symptomatic. Although peripheral nervous system tumors are common in NF1 patients, these patients are also at increased risk of developing pilocytic astrocytomas and, more rarely, malignant astrocytomas. The protein product of the *NF1* gene, located on the long arm of chromosome 17, is neurofibromin 1 (NF1), which functions to antagonize the proliferative function of p21(ras). Neurofibromatosis type 2 (NF2) is distinct from NF1, affecting approximately one in 50,000 individuals.[5] The *NF2* gene, at 22q12.2, encodes a protein known as neurofibromin 2 or merlin (NF2). NF2 patients frequently present before the third decade of

Table 40-1 Hereditary Syndromes Associated with Brain Tumors

Syndrome	Gene	Chromosomal Location	Associated Central Nervous System Tumors
Neurofibromatosis type 1	*NF1*	17q	Optic glioma
Neurofibromatosis type 2	*NF2*	22q12	Acoustic schwannomas, meningioma, ependymoma, glial tumors
Retinoblastoma	*RB*	13q14	Retinoblastoma, pinealoblastoma
Li-Fraumeni syndrome	*P53*	17p13	Glioma, medulloblastoma
von Hippel-Lindau syndrome	*VHL*	3p	Retinal angioma, cerebellar hemangioblastoma
Tuberous sclerosis	*TSC1* *TSC2*	9q 16p13	Giant cell subependymal astrocytoma, astrocytoma, ependymoma
Turcot syndrome	*APC, hMSH1, hMSH2, hPMS2*	5q21	Astrocytoma, medulloblastoma
Gorlin syndrome	*PTCH1*	9q22.1-q31	Medulloblastoma, astrocytoma
Ollier disease	*IDH1, IDH2*	2q33.3, 15q26.1	Endochondroma, rarely glioma

life with deafness caused by bilateral schwannomas of the eighth cranial nerve. Less commonly NF2 patients develop neuronal schwannoma including tumors of the spinal and cranial nerves, meningioma, low-grade astrocytoma, and ependymoma. The Li-Fraumeni syndrome is observed in patients with germline *TP53* mutations.[6] Although affected family members are predisposed to a number of different tumor types, brain tumors are among the more common and include astrocytomas, medulloblastomas, and choroid plexus tumors. The TP53 protein product, p53, plays an important role in the DNA damage checkpoint of cells and in regulating apoptosis. Its function is commonly inactivated by mutation in a wide variety of human tumors and is thought to play an important role in sporadically occurring astrocytic tumors.

Molecular Biology of the Most Common Primary Central Nervous System Tumors

Brain tumors are thought to arise as the result of genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes (Figure 40-1, and see Table 40-1). The precise cell of origin in which the most common brain tumors arise is unknown, but emerging evidence suggests that the malignant transformation of different cell types can give rise to high-grade glioma. There is convincing evidence in animal models of glioma that tumors can arise from the malignant transformation of neural stem cells,[7] very early lineage-specific precursor cells,[8] and more differentiated cells that dedifferentiate[9] and take on key characteristics of precursor cells such as proliferative potential, migratory capacity, and multipotentiality. Identifying the cell type in which tumors arise may provide insight into critical pathologic pathways used by tumor cells. Mouse models of glioma provide strong evidence for the possibility of multiple different cells being targets for malignant transformation, resulting in high-grade glioma.

Significant recent progress has also been made in understanding the cell biology of high-grade glial tumors. An emerging body of data indicates that there is a cellular subpopulation in human glioma, typically consisting of very rare cells, that is distinguished from other tumor cells by the ability to grow in vitro in suspension as neurospheres and to recapitulate the tumor of origin when inoculated orthotopically into immunosuppressed mice.[10] This capacity for tumor initiation is typically not shared with the overwhelming majority of tumor cells, and in contrast to other strategies for immortalizing human glioma, tumor cells grown as neurospheres tend to retain a larger number of the genetic alterations present in the tumors from which they are derived. Study of these tumor-initiating cells, sometimes referred to as tumor stem cells, indicates that they exhibit increased resistance to both radiation and chemotherapy, making them a compelling target for the development of future therapeutic interventions.

Astrocytic Tumors

Diffusely infiltrating astrocytomas are so named because they display morphologic and some biochemical evidence of astroglial differentiation. These are the most common tumors of adults and children, occur throughout the CNS, and exhibit a wide range of histopathologic appearances and clinical behaviors. These tumors are organized by the World Health Organization (WHO) according to tumor grade.[11] Histologically, malignancy is manifested as hypercellularity, cellular atypia, endothelial proliferation, necrosis, and

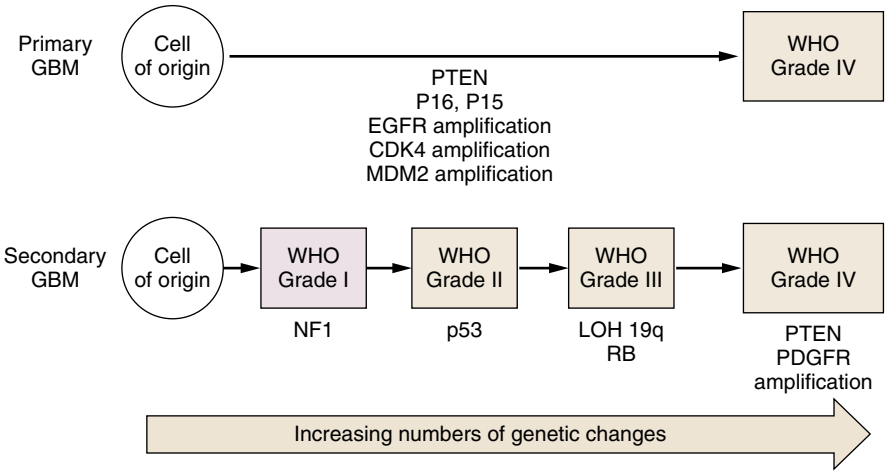


Figure 40-1 Glioblastoma arises in association with the accumulation of multiple genetic alterations. Primary glioblastoma manifests without the presentation of any precursor lesion. Secondary glioblastoma results from the accumulation of additional oncogenic mutations by lower grade astrocytic tumors resulting in increased malignancy. *GBM*, Glioblastoma multiforme; *WHO*, World Health Organization.

invasion of normal adjacent tissue. WHO grade I tumors are variants of astrocytoma that are generally benign, and WHO grade IV, also known as glioblastoma multiforme (GBM), is the most aggressively malignant. Prognosis is closely associated with pathologic grade. WHO grades II and III exhibit intermediate grades of malignancy, but the evidence of increased mitotic activity in grade III tumors is likely to be a key contributor to poor prognosis. Although low-grade tumors can exhibit a circumscribed growth pattern, all pathologic grades of glioma can exhibit invasiveness, and this characteristic compromises the possibility of treating these tumors with surgery alone. The known propensity of some astrocytic tumors that present as low-grade tumors to progress over time to higher grade tumors has provided insight into the pathogenesis of these tumors, suggesting that they are closely related and that progression is associated with the acquisition of sequential genetic alterations (see Figure 40-1; Table 40-2). High-grade astrocytic tumors, GBMs that arise in this manner, are called secondary GBMs. The finding of selected genetic changes (e.g., *TP53* mutation) in lower grade tumors that are also present in higher grade tumors along with additional mutations typically found only in higher grade tumors (e.g., epidermal growth factor receptor [EGFR] amplification) suggests that specific genetic alterations are associated with particular pathologic features characteristic of the corresponding grade. Genetic changes currently thought to be important in this regard are shown in Figure 40-1 and Table 40-2, an adaptation of the pathogenesis model first proposed for colon cancer.[12]

Mutation of the *TP53* gene is likely to be an early event associated with the change of normal cells to low-grade neoplasia. Commonly mutated residues are codons 248 and 273.[13] TP53 has an important role in stabilizing the genome, and the genetic instability resulting from its loss may contribute to the accumulation of multiple mutations in a single cell that are required for the development of highly malignant tumors. Inactivation of *TP53* by mutation or epigenetic mechanisms may occur in up to 75% of astrocytomas. *MDM2*, encoding MDM2, a protein that inhibits the ability of p53 to promote transcription by targeting the protein for degradation, is amplified in approximately 10% of gliomas, and these invariably have a wild-type *TP53* gene. A second gene that is also likely to be mutated early in gliomagenesis is *IDH1*. Mutations of *IDH1* were first identified in studies sequencing glioma cell genomes.[14] Mutations of *IDH1* are found both in lower grade glioma and in GBM. *IDH1* encodes isocitrate dehydrogenase, a Krebs cycle enzyme, and its role in tumorigenesis is an area of intense investigation.

Loss of heterozygosity (LOH) of Ch9p21 at the site of the *CDKN2A* and *CDKN2B* loci leads to homozygous

Table 40-2 Tumor Suppressor Genes and Proto-oncogenes Implicated in Brain Tumor Development

Tumor Suppressor Gene		Proto-oncogene	
Gene or Locus	**Chromosomal Location**	**Gene or Locus**	**Chromosomal Location**
VHL	3p25.3	*EGFR*	7p12
TSC1, TSC2	9q34, 16p13.3	*PDGFRA*	5q31-q32
PTCH1	9q22.3	*MDM2*	12q14
REST	Chr. 4	*HRAS, NRAS*	11p15, 1p13
CDKN2A, CDKN2B	9p21	*CMYC, NMYC*	8q24, 2p23-24
P53	17p13.1		
NF2	22q12		
NF1	17q11.2		
RB1	13q14		
APC	9q31		
PTEN	10q23		
IDH1, IDH2	2q33.3, 15q26		

deletion of these adjacent genes in approximately 60% of GBM. This deletion results in the loss of the p15 (INK4B), p16 (INK4A), and p14 (ARF) tumor suppressor proteins. *CDKN2A* and *CDKN2B* encode cyclin-dependent kinase inhibitors, p16 (INK4A) and p15 (INK4B), respectively, which bind to the cyclin-dependent protein kinases CDK4 and CDK6 inhibiting the catalytic activity of the cyclin D–CDK complex. p1 (ARF) is expressed from a distinct transcript as the result of alternative splicing of the *CDKN2A* gene and functions to keep MDM2 in the nucleolus so that it cannot degrade p53. Loss of p14 (ARF) results in the enhanced degradation of p53. Other cytogenetic changes including +7p/q, +19q, and −1p/q are widely recognized in high-grade brain tumors, but the best understood is clearly the deletion of chromosome 10, where *PTEN* is located. The *PTEN* protein product, PTEN, is a lipid phosphatase that antagonizes the function of the phosphatidylinositol-3-kinase (PI3K) family of lipid messengers and consequently inhibits downstream signaling through AKT1, a serine/threonine kinase that is a key regulator of critical cell functions including cell proliferation and survival.

Several different receptor tyrosine kinases (RTKs) and their cognate ligands have been implicated in the malignant behavior of astrocytic tumors, and especially GBM. These include PDGF/PDGFR, EGF and TGF-α/EGFR, IGF/IGFR, and others. Although amplification of *EGFR* is found in approximately 50% of GBMs, this change is rarely found in WHO grade III and never in grade I or II tumors. Also, small deletions and rearrangements of the *EGFR* gene are commonly found in GBM.[15] As many as 50% of tumors in which amplification of *EGFR* is detectable also have a rearrangement of *EGFR* in which exons 2 to 7 are deleted.[16] This results in an in-frame deletion that encodes a constitutively active *EGFR* (*EGFRvIII*), which, along with EGFR amplification in tumor cells, predicts a poor outcome. Both antibodies and small-molecule therapeutics have been used to target EGFR in glioma, but resistance to such therapies is rapidly manifested when these treatments are used alone. Although members of the PDGF pathway tend to be overexpressed in all grades of astrocytic tumors, amplification and mutation are rare. Activation of RTKs in glioma leads to autophosphorylation of these receptors, which in turn leads to the docking of a series of proteins on phosphorylated sites of the receptors and downstream activation of various effectors. Activation of RAS by the docking protein SOS1 leads to initiation of the RAF/MEK/MAPK (ERK) pathway that leads to the transcriptional activation of genes important for proliferation. Docking of GAB1 to another phosphorylated site on autophosphorylated RTKs leads to activation of PI3K, which in turn initiates a cascade of phosphorylation leading to PDK1 phosphorylation of AKT1. Activated AKT1 in turn phosphorylates both a number of pro-apoptotic proteins, inactivating them, and a series of transcription factors, activating and leading to transcription of other genes important for cellular proliferation. About 80% of GBMs exhibit activation of AKT1, primarily as a result of RTK activation or deletional inactivation of *PTEN*.[17]

Table 40-3 Glioblastoma Multiforme Subtypes as Suggested by Verhaak et al.

Genes Mutated in Glioblastoma	Glioblastoma Multiforme Subtypes (Frequency of Mutation)			
	Proneural	Neural	Classical	Mesenchymal
TP53	54%	21%	0%	12%
NF1	5%	16%	5%	37%
EGFR	16%	5%	0%	0%
EGFRvIII	3%	0%	23%	3%
IDH1	30%	5%	0%	0%
PDGFRA	11%	0%	0%	0%

Data from Verhaak RG et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010;17:98-110.

It is now widely recognized that not all GBMs exhibit the same constellation of genetic alterations, and, interestingly, a second clinical presentation of GBM seems to be associated with a distinctive genetic profile (see Figure 40-1; Table 40-3). Approximately 90% of patients with GBMs present without evidence of a precursor lesion (de novo, primary GBM). Although high levels of *EGFR* expression are frequently found in primary GBM, these are rarely detectable in secondary GBM, which is associated with lower grade precursor lesions. In primary GBM, amplification of *MDM2* is a more common mechanism for inactivation of *TP53* than it is in secondary GBM. The *CDKN2A* and *PTEN* loci are also frequently inactivated by mutation in primary GBM, whereas *IDH1* is more commonly mutated in secondary GBM.

It has not yet been possible to recognize a characteristic pathology or clinical presentation associated with the genetic heterogeneity that is now well documented to accompany the histologic and cytologic variation characteristic of GBM. Current interpretation of the extensive genomic evaluation of glioma gene structure and gene expression suggests that among histologically indistinguishable tumors, there are definable subtypes of glioma characterized by distinctive molecular alterations (see Table 40-3).[18,19] These classifications may be of prognostic significance,[20] although proven targets for therapeutic intervention have not yet been identified.

Oligodendroglioma

Oligodendrogliomas account for about 20% of primary intracranial tumors and are the second most common glial neoplasm.[1] Most frequently these tumors occur in the cerebral hemispheres, typically within the white matter. They are characterized by various histologic criteria of malignancy, which determine their grade. These tumors can have a highly variable spectrum of differentiation, and their cellular origins are not understood, although it is likely that some high-grade gliomas arise from oligodendrocytic precursor cells (OPCs) that are present in the adult nervous system.[21] There is frequent confusion over the proper diagnostic classification for tumors that do not display prototypical characteristics of cells arising in the oligodendroglial lineage or contain multiple populations of cells, one of which will correspond to the oligodendroglial lineage and the other to the astrocytic lineage. Histopathologically, tumors of the oligodendroglial lineage exhibit round, homogeneous, compact cells with a dense central nucleus and clear cytoplasm, sometimes referred to as a "fried egg" appearance. There is rarely identifiable evidence of mitosis. When tumors are highly anaplastic with obvious necrosis and mitotic figures, they are indistinguishable from GBM and are designated as such. Different histologic grades of oligodendroglioma have been associated with specific patterns of gene expression[22] and genetic alterations.[23]

The cytogenetic and molecular biologic profiles of oligodendroglioma are not well characterized but are quite distinct from those of astrocytoma.[23,24] Cytogenetic studies of oligodendroglioma may reveal loss of chromosomes 9p and 22 or gain of chromosome 7, but the most characteristic cytogenetic findings in oligodendroglioma are loss of chromosomes 1p36 and 19q13.3. The presence of these alterations is associated with an enhanced responsiveness to cytotoxic therapies and an improved prognosis.[25] LOH for chromosome 9p, observed in some oligodendrogliomas, corresponds to deletions at the *CDKN2A* locus, and these have been associated with a poor prognosis for patients with this tumor type. Studies looking for mutations that are frequently found in astrocytic tumors have not detected commonly occurring changes in the *TP53* locus. Similarly, Ch10 losses and *EGFR* amplification have been detected only rarely in these tumors. These distinguishing molecular genetic differences are consistent with more recent genomic scale expression analyses that reveal clear differences between astrocytoma and oligodendroglioma.

Meningioma

Meningiomas account for approximately 20% of primary intracranial malignancies[1] and are derived from mesoderm, probably from cells giving rise to the arachnoid granulations. These tumors are usually benign and attached to the dura mater. They infrequently invade the brain parenchyma. Meningiomas may be found incidentally on a computed tomography (CT) or magnetic resonance imaging (MRI) scan, or they may present with a focal seizure, a slowly progressive neurologic deficit, or symptoms of raised intracranial pressure. Total surgical resection of benign meningioma is curative, and when tumor persists, external beam radiotherapy or stereotaxic radiosurgery reduces the recurrence rate to less than 10%. No effective chemotherapy for the treatment of benign meningioma is known, and there is no curative therapy for anaplastic, highly invasive meningioma that cannot be treated locally.

Meningiomas are an important manifestation of neurofibromatosis type 2 (NF2), which occurs as the result of inactivating mutations of the NF2 gene at 22q12. NF2 functions as part of a molecular complex linking the cellular plasma membrane to the cytoskeleton, where it has been implicated in the regulation of cellular proliferation. About half of NF2 patients develop meningioma, and portions of the NF2 gene are lost in approximately half of sporadic meningioma. The frequency of *NF2* mutation does vary somewhat in different histologic types of meningioma.[26,27] *NF2* mutations occur in only about 25% of meningothelial meningiomas, whereas more than 75% of fibroblastic and transitional meningiomas have been found to have evidence of *NF2* inactivation. Other karyotypic abnormalities are also seen in grade II (atypical meningioma) and grade III (anaplastic meningioma). In anaplastic meningioma, the most frequent cytogenetic abnormalities are deletion of Ch1p, partial or complete loss of Ch10q, and loss of Ch14. Unstable chromosome alterations including rings, dicentrics, and telomeric associations also have been observed.

Medulloblastoma

Among children with cancer, only leukemia is more common than primary brain tumors. Medulloblastoma accounts for approximately 15% to 20% of all pediatric brain tumors and for about 80% of childhood primitive neuroectodermal tumors (PNETs) of the CNS. Only gliomas are more common in children. In contrast to other PNET, these tumors are found in the cerebellum, where they are thought to arise from neural progenitor cells (NPCs) of the fetal external granular layer.[28] The 2007 WHO classification of CNS tumors recognized several histologic subtypes of medulloblastoma,[11] and preliminary studies suggest that these are of prognostic significance. Atypical teratoid/rhabdoid tumors of the CNS identified by somatic mutations of the *INI1*

gene often occur in the posterior fossa and can be difficult to distinguish from medulloblastoma. In contrast, medulloblastoma is readily distinguishable from childhood glioma in that it appears as a highly cellular, small, round-cell tumor with frequent mitoses and pseudorosettes. Also, medulloblastoma is more responsive to treatment than glioma, as evidenced by the importance of therapeutic response as a prognostic factor in this disease.[29] The 5-year disease-free survival rate approximates 50% to 70%.[30]

As noted in Table 40-1, the occurrence of medulloblastoma in three inherited cancer syndromes—Turcot syndrome, Li-Fraumeni syndrome, and Gorlin syndrome—has called attention to the possibility that the genes responsible for these syndromes, *APC*, *TP53*, and *PTCH1*, respectively, or the pathways in which they are active might play a role in sporadic medulloblastoma. Although evidence for APC inactivation in sporadic medulloblastoma has not been identified, *TP53* mutations and mutations of *PTCH1* and genes in the *PTCH1*-mediated sonic hedgehog (SHH) pathway are found in sporadically occurring medulloblastoma.[31] Deletions of Ch6q and Ch16q have also been noted in medulloblastoma tumor tissues, and infrequent amplification of *MYC* and *MYCN* has been detected. *NTRK3* neurotrophin receptor expression in medulloblastoma has been reported to be associated with a favorable outcome,[32] a finding consistent with emerging evidence that medulloblastoma tumor tissue expresses a number of genes associated with the neuronal lineage and that expression profiling of these genes may provide a particularly effective approach to devising clinically useful prognostic biomarkers.[33,34]

Molecular Pathophysiology of Primary Brain Tumors

Brain tumors present therapeutic challenges that reflect their occurrence within the closed space of the skull and within a tissue that is particularly sensitive to disruption of its normal function. As a result, simple growth as a space-occupying lesion is particularly problematic in the development of brain tumors as is the invasion of normal tissue, which can occur early in the pathogenesis of some tumor types such as primary GBM and much later in others. The proliferation of brain tumors is thought to be the result of the deregulation of oncogenes and tumor suppressor genes as described previously, and many of the growth-stimulatory pathways that contribute to tumorigenesis in the CNS have been extensively examined. In addition to the oncogenetic alterations described for individual tumors, important regulatory roles in the proliferation of brain tumors include those mediated by EGF and PDGF, which are widely expressed in the normal brain as well.

Glioma and medulloblastoma are typically highly invasive. This characteristic is thought to play an important role in their recurrence after treatment, which tends to occur locally at their original site of presentation. The mechanisms that underlie glial tumor cell invasion are an active area of research.[35-37] Clinicians observed long ago that such malignant tumors have preferred routes of invasion along white matter tracts—although little is known of the manner in which tumor cells recognize white matter tracts and migrate along them. Integrins, especially AvB3 and AvB5, seem to play an important role in this process.[38] Degradation of the extracellular matrix by proteinases produced by tumor cells is now recognized as an important early step in the invasion of normal tissue. Glial tumor cells express urokinase-type plasminogen receptor (UPAR) on their surface,[39] which binds to urokinase plasminogen activators (UPAs) that are also highly expressed in the most malignant gliomas.[39] UPAR presents activated UPA to the extracellular matrix (ECM), where plasminogen is cleaved to active plasmin. This degrades ECM constituents including fibronectin, laminin, and proteoglycans that are especially prominent in the ECM surrounding blood vessels. Matrix metallopeptidases, especially MMP2 and MMP9, also seem to play important roles in ECM degradation.[40] Interestingly, some brain tumors produce inhibitors of various plasminogen activators, thereby inhibiting ECM degradation. The extracellular milieu of the CNS also contains high concentrations of glycosaminoglycan, hyaluronan, and chondroitin sulfate, which are detected in only low amounts in other tissues. How brain tumor cells move through a matrix of such molecules is not known.

The development of a tumor vasculature is a central aspect of tumorigenesis and tumor progression.[41] Although low-grade brain tumors tend to have a vasculature similar to that observed in normal brain, high-grade tumors are heavily vascularized and disrupt the established blood-brain barrier. Such a barrier is not present in the new tumor vessels that are formed.[42] Glial tumor vessel formation is regulated by the endothelial cell RTKs for VEGF, including FLT-1 (VEGFR1) and KDR (VEGFR2).[43] VEGF is a critical mediator of tumor angiogenesis in several different types of brain tumors, and its expression is inducible by hypoxia, a finding consistent with cellular hypoxia being a key stimulant for angiogenesis during glioma formation. VEGF is expressed at very high levels in high-grade gliomas.[44] FLT-1 is not expressed in normal brain tissue but is expressed in a tumor-stage–dependent manner in the vasculature of astrocytoma, and *KDR* is expressed only in high-grade glioma.[43] VEGF is also known to enhance the vascular permeability of normal brain vasculature and may be responsible for the peritumoral edema found frequently in malignant gliomas. Blood vessel formation during tumorigenesis and tumor

progression requires both proliferation and recruitment of smooth muscle pericytes, which is regulated by TGFB1, PDGFRB, and TEK (TIE-2). The TEK receptor and its agonist, ANGPT1, are constitutively expressed in gliomas, suggesting an important role in the development of tumor vasculature for this pathway as well. Bevacizumab, an antibody against VEGF, is widely used in the management of brain tumor patients, though the degree of impact on survival and quality of life remains unclear.[45]

Therapeutic Resistance of Primary Central Nervous System Tumors

Primary brain tumors are widely regarded as being particularly resistant to the most commonly used antineoplastic strategies. Although surgery plays a major role in removing some brain tumors and debulking others, the challenges of working within the skull in a tissue so exquisitely sensitive to anatomic disruption means that tumors that either arise in or invade regions of the brain that are required for vital functions cannot be effectively removed. Also, aggressive brain tumors, especially high-grade astrocytomas, tend to invade as numerous projections from the surface of the tumor and as rare cells that move to very distant regions of the brain, making complete removal impossible. Similarly, the use of radiation therapy is challenged by the compactness of normal tissues in the vicinity of the tumor and by the invasion of tumor into normal tissue. These characteristics of brain tumors mean that delivering effective doses of radiation to the tumor without damaging critical normal tissue is particularly difficult. This is especially important in the developing brains of pediatric patients and compromises our ability to use the highest doses of radiation therapy in children.

Both radiation therapy and chemotherapy are compromised because many glial-derived tumors seem to be particularly resistant to apoptosis following DNA damage.[46] Although the precise contribution of apoptosis to curative therapy in solid tumors is not yet known, it is clear that tumors that are highly resistant to apoptosis do not respond well to cytotoxic therapies. Also, the blood-brain barrier presents a unique challenge for the administration of chemotherapy. Although the barrier is largely destroyed at sites of primary tumor growth, migrating, invasive tumor cells are thought to remain behind the blood-brain barrier until they begin to establish millimeter-size masses, and this finding may account for the apparent resistance to therapy of some tumor types. The use of combination therapy consisting of procarbazine, lomustine (CCNU), and vincristine (PCV) has been found to be quite active in a subgroup of patients with oligodendroglioma, which is characterized by the loss of Ch1p and Ch19q.[47] Also, the combination of temozolomide with radiation has resulted in a slight increase in median survival of patients with GBM, a disease in which surgery and radiation alone result in survival of approximately 1 year.[48] These results indicate that cytotoxic approaches can contribute to the treatment of brain tumors and, it is hoped, will provide insights into how multimodality therapy might be more effective in the future.

Future Directions/Perspective

Recent advances in understanding the genetic underpinnings of primary brain tumors have provided evidence for the involvement of genes and pathways not previously known to be important for the pathobiology of glioma, and in some cases for any cancer. These studies have provided a remarkable picture of the molecular heterogeneity that previous work suggested, and many expected, but is now irrefutable and clearly of great importance in thinking about the development of future therapies. Beyond the mutational heterogeneity important for tumor progression and recurrence, the documentation of epigenetic, biological, and molecular heterogeneity in different parts of high-grade glioma[3]—an observation consistent with the histologic appearance of GBM—raises important questions regarding the design and evaluation of current therapeutic strategies. Characterizations of these genetic and epigenetic changes associated with malignancy have highlighted pathways likely to be the "drivers" of tumor pathology and tumor progression. Although the failure of targeted therapies has to date been focused on target-specific mutations contributing to therapeutic resistance,[49] we will now have to contend with large populations of cells within tumors at presentation and relapse that may require quite different therapeutic approaches[3] and tumor stem cell populations that are intrinsically chemoresistant.[50] Importantly, the need for combinations of targeted therapy is further highlighted by these observations, and that clearly should be an important line of clinical investigation for the immediate future.

Key References

1. Howlader N, et al. SEER *Cancer Statistics Review, 1975-2009*. Bethesda, Md: National Cancer Institute; April 2012. Based on November 2011 SEER data submission http://seer.cancer.gov/csr/1975_2009_pops09/.
2. Surveillance, Epidemiology, and End Results (SEER) Program. Surveillance Research Program, Surveillance Systems Branch, April 2012, based on November 2011 submission (www.seer.cancer.gov).
3. Nickel GC, et al. Characterizing mutational heterogeneity in a glioblastoma patient with double recurrence. *PLoS One*. 2012;7:e35262.
7. Zhu Y, et al. Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma. *Cancer Cell*. 2005;8:119-130.
8. Liu C, et al. Mosaic analysis with double markers reveals tumor cell of origin in glioma. *Cell*. 2011;146:209-221.
10. Singh SK, et al. Identification of human brain tumour initiating cells. *Nature*. 2004;432:396-401.
11. Louis DN, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*. 2007;114:97-109.
13. Stander M, et al. Prognostic impact of TP53 mutation status for adult patients with supratentorial World Health Organization Grade II astrocytoma or oligoastrocytoma: a long-term analysis. *Cancer*. 2004;101:1028-1035.
14. Parsons DW, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807-1812.
16. Aldape KD, et al. Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. *J Neuropathol Exp Neurol*. 2004;63:700-707.
17. Wang H, et al. Analysis of the activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas. *Lab Invest*. 2004;84:941-951.
18. Verhaak RG, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17:98-110.
19. Liang Y, et al. Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. *Proc Natl Acad Sci U S A*. 2005;102:5814-5819.
20. Phillips HS, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell*. 2006;9:157-173.
21. Persson AI, et al. Non-stem cell origin for oligodendroglioma. *Cancer Cell*. 2010;18:669-682.
23. Bettegowda C, et al. Mutations in CIC and FUBP1 contribute to human oligodendroglioma. *Science*. 2011;333:1453-1455.
26. Buccoliero AM, et al. NF2 gene expression in sporadic meningiomas: relation to grades or histotypes real time-pCR study. *Neuropathology*. 2007;27:36-42.
35. Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. *J Neurooncol*. 2004;70:217-228.
36. Hoelzinger DB, et al. Gene expression profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. *Neoplasia*. 2005;7:7-16.
38. D'Abaco GM, Kaye AH. Integrins: molecular determinants of glioma invasion. *J Clin Neurosci*. 2007;14:1041-1048.
45. Chamberlain MC. Bevacizumab for the treatment of recurrent glioblastoma. *Clin Med Insights Oncol*. 2011;5:117-129.
46. Lefranc F, Brotchi J, Kiss R. Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. *J Clin Oncol*. 2005;23:2411-2422.
48. Stupp R, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987-996.
49. Taylor TE, Furnari FB, Cavenee WK. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr Cancer Drug Targets*. 2012;12:197-209.
50. Beier D, Schulz JB, Beier CP. Chemoresistance of glioblastoma cancer stem cells—much more complex than expected. *Mol Cancer*. 2011;10:128.

References

1. Howlader N, et al. SEER *Cancer Statistics Review, 1975-2009*. Bethesda, Md: National Cancer Institute; 2012. Based on November 2011 SEER data submission http://seer.cancer.gov/csr/1975_2009_pops09/.
2. Surveillance, Epidemiology, and End Results (SEER) Program. Surveillance Research Program, Surveillance Systems Branch, April 2012, based on November 2011 submission (www.seer.cancer.gov).
3. Nickel GC, et al. Characterizing mutational heterogeneity in a glioblastoma patient with double recurrence. *PLoS One*. 2012;7:e35262.
4. Listernick R, Charrow J, Gutmann DH. Intracranial gliomas in neurofibromatosis type 1. *Am J Med Genet*. 1999;89:38-44.
5. Martuza RL, Eldridge R. Neurofibromatosis 2 (bilateral acoustic neurofibromatosis). *N Engl J Med*. 1988;318:684-688.
6. Varley JM, et al. Germ-line mutations of TP53 in Li-Fraumeni families: an extended study of 39 families. *Cancer Res*. 1997;57:3245-3252.
7. Zhu Y, et al. Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma. *Cancer Cell*. 2005;8:119-130.
8. Liu C, et al. Mosaic analysis with double markers reveals tumor cell of origin in glioma. *Cell*. 2011;146:209-221.
9. Bachoo RM, et al. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. *Cancer Cell*. 2002;1:269-277.
10. Singh SK, et al. Identification of human brain tumour initiating cells. *Nature*. 2004;432:396-401.
11. Louis DN, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*. 2007;114:97-109.
12. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61:759-767.
13. Stander M, et al. Prognostic impact of TP53 mutation status for adult patients with supratentorial World Health Organization Grade II astrocytoma or oligoastrocytoma: a long-term analysis. *Cancer*. 2004;101:1028-1035.
14. Parsons DW, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science*. 2008;321:1807-1812.
15. Frederick L, et al. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res*. 2000;60:1383-1387.
16. Aldape KD, et al. Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. *J Neuropathol Exp Neurol*. 2004;63:700-707.
17. Wang H, et al. Analysis of the activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas. *Lab Invest*. 2004;84:941-951.
18. Verhaak RG, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17:98-110.
19. Liang Y, et al. Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. *Proc Natl Acad Sci U S A*. 2005;102:5814-5819.
20. Phillips HS, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell*. 2006;9:157-173.
21. Persson AI, et al. Non-stem cell origin for oligodendroglioma. *Cancer Cell*. 2010;18:669-682.
22. Watson MA, et al. Gene expression profiling with oligonucleotide microarrays distinguishes World Health Organization grade of oligodendrogliomas. *Cancer Res*. 2001;61:1825-1829.
23. Bettegowda C, et al. Mutations in CIC and FUBP1 contribute to human oligodendroglioma. *Science*. 2011;333:1453-1455.
24. Reifenberger J, et al. Molecular genetic analysis of oligodendroglial tumors shows preferential allelic deletions on 19q and 1p. *Am J Pathol*. 1994;145:1175-1190.
25. Cairncross JG, et al. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst*. 1998;90:1473-1479.
26. Buccoliero AM, et al. NF2 gene expression in sporadic meningiomas: relation to grades or histotypes real time-pCR study. *Neuropathology*. 2007;27:36-42.
27. Wellenreuther R, et al. Quantitative analysis of neurofibromatosis type 2 gene transcripts in meningiomas supports the concept of distinct molecular variants. *Lab Invest*. 1997;77:601-606.
28. Marino S, et al. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev*. 2000;14:994-1004.
29. Packer RJ, et al. Outcome for children with medulloblastoma treated with radiation and cisplatin, CCNU, and vincristine chemotherapy. *J Neurosurg*. 1994;81:690-698.
30. McNeil DE, et al. Incidence and trends in pediatric malignancies medulloblastoma/primitive neuroectodermal tumor: a SEER update. Surveillance Epidemiology and End Results. *Med Pediatr Oncol*. 2002;39:190-194.
31. Reifenberger J, et al. Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. *Cancer Res*. 1998;58:1798-1803.
32. Grotzer MA, et al. TrkC expression predicts good clinical outcome in primitive neuroectodermal brain tumors. *J Clin Oncol*. 2000;18:1027-1035.
33. Lawinger P, et al. The neuronal repressor REST/NRSF is an essential regulator in medulloblastoma cells. *Nat Med*. 2000;6:826-831.
34. Tohyama T, et al. Nestin expression in embryonic human neuroepithelium and in human neuroepithelial tumor cells. *Lab Invest*. 1992;66:303-313.
35. Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. *J Neurooncol*. 2004;70:217-228.
36. Hoelzinger DB, et al. Gene expression profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. *Neoplasia*. 2005;7:7-16.
37. Tysnes BB, Mahesparan R. Biological mechanisms of glioma invasion and potential therapeutic targets. *J Neurooncol*. 2001;53:129-147.
38. D'Abaco GM, Kaye AH. Integrins: molecular determinants of glioma invasion. *J Clin Neurosci*. 2007;14:1041-1048.
39. Yamamoto M, et al. Expression and localization of urokinase-type plasminogen activator in human astrocytomas in vivo. *Cancer Res*. 1994;54:3656-3661.
40. Forsyth PA, et al. Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. *Br J Cancer*. 1999;79:1828-1835.
41. Folkman J. Tumor angiogensis: role in regulation of tumor growth. *Symp Soc Dev Biol*. 1974;30:43-52.
42. Vajkoczy P, Menger MD. Vascular microenvironment in gliomas. *J Neurooncol*. 2000;50:99-108.
43. Plate KH, et al. Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms. *Int J Cancer*. 1994;59:520-529.
44. Chan AS, et al. Expression of vascular endothelial growth factor and its receptors in the anaplastic progression of astrocytoma, oligodendroglioma, and ependymoma. *Am J Surg Pathol*. 1998;22:816-826.
45. Chamberlain MC. Bevacizumab for the treatment of recurrent glioblastoma. *Clin Med Insights Oncol*. 2011;5:117-129.
46. Lefranc F, Brotchi J, Kiss R. Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. *J Clin Oncol*. 2005;23:2411-2422.
47. Levin VA. Chemotherapy for brain tumors of astrocytic and oligodendroglial lineage: the past decade and where we are heading. *Neuro Oncol*. 1999;1:69-80.
48. Stupp R, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352:987-996.
49. Taylor TE, Furnari FB, Cavenee WK. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr Cancer Drug Targets*. 2012;12:197-209.
50. Beier D, Schulz JB, Beier CP. Chemoresistance of glioblastoma cancer stem cells—much more complex than expected. *Mol Cancer*. 2011;10:128.

Adam B. Glick and Stuart H. Yuspa

41

Epithelial Skin Cancer

Skin Cancer Research Has Helped Define the Biology of Cancer Pathogenesis

Epithelial nonmelanoma skin cancer (NMSC, primarily basal cell and squamous cell carcinoma) is the most frequent cancer among white populations, with incidence rates matching all other cancers combined in these groups. Although not frequently lethal, epithelial skin cancers are the cause of considerable morbidity, cosmetic defects, and extreme medical expense. Exposure to mutagenic ultraviolet (UV) light is the major skin carcinogenetic stimulus, but sun avoidance has often been superseded by lifestyle choices, and changing atmospheric conditions favor increased intensity of exposure. Thus, considerable attention has been directed to understanding the molecular pathogenesis of epithelial cutaneous cancer, and much progress has been achieved. Consequently, effective therapeutic approaches are evolving that have the potential to sharply reduce the morbidity and expense associated with these lesions. Skin cancer research has also provided significant insight into the mechanisms of understanding cancer pathogenesis in general. Centuries ago, astute clinicians and basic scientists first observed that chemical and physical exposures could cause skin cancer in humans and animals. Modern analysis of skin cancer induction demonstrated that cancers evolve from multiple, clonal, precancerous stages (initiation, promotion, premalignant progression, malignant conversion), and that genetic (e.g., mutation), epigenetic (e.g., gene expression or protein modification), and microenvironmental changes (e.g., inflammation, wounding) contribute to tumor formation. Skin studies first revealed that chemical carcinogens bind covalently to DNA, causing specific mutations in oncogenes and suppressor genes, and that DNA repair protects against cancer risk. In addition, cutaneous cancer research has indicated that viruses can cause human tumors; that immunosuppressed patients are susceptible to cancer; that genetic background can modify cancer rates; that diet, UV exposure, and cigarette smoke are cancer risk factors; and that signaling pathways initially associated with familial basal cell carcinoma (BCC) contribute to the pathogenesis of a variety of epithelial cancers of internal organs.[1] Thus, epithelial skin cancer research has been a major contributor to defining cancer biology and biochemistry in general.

The Molecular Origin of Skin Tumors Is Revealed by Hereditary Syndromes

Cutaneous cancers are the manifestation of germline mutations in a number of hereditary syndromes (Table 41-1). Identifying the mutant genes has revealed pathways relevant to the pathogenesis of cancers in skin and internal organs as well as revealing targets for somatic mutations in spontaneous versions of the same tumor type. The most broadly relevant are the DNA repair genes that comprise the complementation groups of skin cancer–prone xeroderma pigmentosum families.[2] Germline mutations in individual genes at distinct chromosomal loci define proteins that collectively ensure proper global and transcription-coupled nucleotide excision repair. Among these are proteins that recognize and bind to sites of DNA damage (XPA, XPC, XPE),[1] helicases (XPB, XPD), and endonuclease components (XPG, XPF)—defects in any of which can give a skin cancer—prone or defective neurological phenotype.[2] The discovery of germline mutations in the *PTCH1* gene among patients with the basal cell nevus syndrome first revealed the involvement of the Sonic hedgehog pathway in human cancer.[3] Subsequent studies confirmed that most sporadic basal cell cancers had somatic mutations in *PTCH1* or its downstream effector *SMO*. The mapping of the dysplastic nevus syndrome to the *INK4a* locus and identification of mutations in the p16*(INK4a)* gene revealed an important pathway in the etiology of heredity-prone and sporadic melanoma. As important, this discovery pointed to defects in structure or expression of p16 *(INK4a)* and other components of the cyclin-CDK signaling pathway in melanoma and

Table 41-1 Gene Targets for Mutations in Hereditary and Sporadic Cutaneous Cancers

Gene	Function	Locus	Tumor Type	Syndrome	Spontaneous
p53	DNA repair, apoptosis, cell cycle regulation	17 p13.1	BCC, SCC	Li-Fraumeni (but no increase in skin cancers)	Yes
XPA, XPB XPC, XPD, XPE XPF, XPG	DNA repair	9p34.1, 2q21 3p25.1, 19q13.3, 11p11.12 16p13.3, 13q32	BCC, SCC, melanoma	Xeroderma pigmentosum	Possible
PTCH1	Sonic hedgehog receptor	9q22.3	BCC, trichoepithelioma	Nevoid basal cell carcinoma	Yes
SMO	Sonic hedgehog effector	7q31-32	BCC	Not identified	Yes
$p16^{INK4a}$	Cyclin inhibitor	9p21	Melanoma, SCC, trichoepithelioma	Dysplastic nevus	Yes
CTNNB (β-catenin)	Cell-cell adhesion, transcription factor	3p22-p21.3	Pilomatricoma	Not identified	Yes
CYLD1	NFκB inhibitor	16q12-13	Cylindroma Trichoepithelioma	Multiple cylindroma	Yes
PTEN	Phosphatase	10q23.3	Trichilemmoma	Cowden's	Unknown
MSH2 MLH1	Mismatch repair	2p22-p21 3p21.3	Sebaceous gland carcinoma	Muir-Torre	Unknown
Folliculin	Regulates mTor	17p11.2	Fibrofolliculoma	Birt-Hogg-Dube	Unknown
CYLD1	NFκB inhibitor	16q12-13	Trichoepithelioma	Multiple trichoepithelioma	Unknown
?	?	Xq24-q27	BCC	Bazex	Unknown
TGFβR1	Growth inhibitor	9q22.3	Keratoacanthoma	Ferguson-Smith	Iatrogenic
DKC1, TERC, others	Telomere maintenance	X, 3q, others	SCC	Dyskeratosis congenita	No
COL7A1	Anchoring fibrils	3p21.1	SCC	Recessive dystrophic epidermolysis bullosa	No

nonmelanoma skin cancer. Ferguson-Smith disease (FSD), characterized by multiple squamous-carcinoma–like locally invasive skin tumors that grow rapidly for a few weeks and then spontaneously regress, is due to inactivating mutations in *TGFβR1*. Recessive dystrophic epidermolysis bullosa is a blistering skin disorder caused by mutations in *COL7A1*, a critical component of the basement membrane secreted by keratinocytes. DEBS patients suffer from early-onset, highly invasive and metastatic squamous cell carcinoma (SCC), often leading to death. Tumorigenesis is linked to a specific fragment of type VII collagen that promotes tumor cell invasion and constitutively active dermal fibroblasts that enhance tumor aggressiveness. Specific mutations in Cowden syndrome (*PTEN*), Muir-Torre syndrome (*MSH2, MLH1*), pilomatricoma (*CTNNB [β-catenin]*), Birt-Hogg-Dube syndrome (*FLCN*), cylindromatosis (*CYLD1*), and trichoepithelioma (*PTCH1*, p16 (*INK4aA*)) that cause adnexal tumors have revealed much about the pathways important in the development of cutaneous adnexal tissues. In addition to skin tumors, folliculin defects are strongly linked to renal cancer, Pten to breast cancer, and Msh2 to colon cancer. Dyskeratosis congenita is a complex genetic syndrome resulting from defects in any one of related genes involved in the maintenance of telomeres. The association with both cutaneous and head and neck SCC recapitulates mouse models of telomerase deficiency.

In addition to these highly penetrant hereditary syndromes, genome-wide association studies are identifying new loci that confer increased risk for either BCC or SCC, although the mechanistic details remain. Single-nucleotide polymorphisms (SNPs) within several well-characterized pigmentation loci—including *MC1R* (melanocortin 1 receptor) and *OCA2* (oculocutaneous albinism II)—and a potential pigmentation locus *IRF4* (interferon regulatory factor 4) show linkage to increased risk for both BCC and SCC NMSC.[4-6] Other SNPs on 6p25 and 13q32 near the *EXOC2* and *UBAC2* genes also show linkage with increased risk of SCC,[6] whereas two functional SNPs in the nucleotide excision repair gene *ERCC6* are significantly associated with increased risk of BCC. A history of NMSC is also associated with increased risk for subsequent noncutaneous malignancies, and a previous SCC is associated with poor prognosis for cancers of the lung, colon, rectum, and breast and for non-Hodgkin lymphoma. Although the genetic basis of this is currently unknown, GWAS studies of patients

with NMSC and a noncutaneous malignancy will likely reveal additional loci conferring risk for cancer for which the appearance of NMSC may be a signal.

Basal Cell Carcinoma

BCC is a very common, slow-growing, locally invasive tumor that typically presents as a pink or pearly papule with superficial telangiectasia and occasional ulceration. BCC precursor lesions have not been identified, there is no evidence of neoplastic progression, phenotypic diversity even within the same tumor is common, and metastases are exceedingly rare. Chromosomal losses involving 9q mark both sporadic and inherited BCCs, leading to the discovery of mutations in *PTCH1*,[3] a homologue of the *Drosophila ptc* gene involved in embryonic development.

Ptch1 is a 12-pass transmembrane receptor for Sonic hedgehog (Shh), a secreted ligand involved in the proliferation and patterning of multiple tissues and organs during embryogenesis. Shh binds to Ptch and alleviates Ptch-mediated repression of Smo on responding cells (reviewed in Reference 1). Active Smo enters the primary cilium, where it functions to modify the expression and structure of a family of Gli transcription factors to alter the expression of Shh target genes. The complexity of the pathway is enhanced by the presence of additional vertebrate homologues of *Drosophila* proteins, fused (a serine-threonine kinase), suppressor of fused (SuFu) that binds to Gli proteins, Cos 2 (Kif7), and Iguana (Dzip1), with SuFu, Cos2, and Iguana serving as inhibitors of Gli signaling.[1] Activation of the Shh pathway upregulates *Gli1* and *Ptch1*, and these serve as markers for physiologic and pathologic Shh signaling. In human BCCs, inactivating mutation or deletion of PTCH1 results in constitutive signaling independent of SHH. In addition to loss-of-function *PTCH1* mutations, gain-of-function (oncogenic) *SMO* mutations have been found in some BCCs where PTCH1 appears to be normal. *PTCH1* or *SMO* mutations are implicated in about 80% of BCCs, whereas hedgehog signaling is active in all BCCs. Thus, additional mechanisms must exist for uncontrolled activation of this pathway. Rare mutations in SuFu have been reported in BCCs, and other syndromes are known to predispose to BCC, but the genetic bases for these have not been delineated. Although mutations in *p53* have been found in up to 50% of BCCs , most lesions fail to exhibit the genomic instability associated with other cancers where p53 function is compromised,[7] and the presence or absence of p53 mutations does not alter the histological phenotype, although loss of p53 function facilitates the eruption of BCC in animal models.

Gli activation is the driving force for tumor development in the setting of constitutive Hedgehog signaling. The three mammalian Gli proteins are zinc finger proteins that bind to a 9bp canonical binding site in the promoters of target genes following activation of hedgehog signaling (reviewed in Reference 1). The regulation of Gli activity by hedgehog signaling is complex; Gli2 and Gli3 proteins have both transcriptional repressor and activating domains, whereas Gli1 is strictly a transcriptional activator. Much of the regulation of Gli proteins is post-translational. Gli3 processing primarily generates a transcriptional repressor, whereas processed (phosphorylated) Gli2 is ubiquitinated and degraded. Shh signaling suppresses processing and degradation of Gli2 and stabilizes its transcriptional activation function. Genetic ablation studies in mice have displayed the consequences of Gli activity in vivo. Gli1-null mice are without a phenotype, whereas the developmental defects and impaired hair follicle growth caused by ablation of Gli2 indicate that Gli2 is the downstream effector of the Hh pathway.[8] Disruption of Gli3 produces a phenotype consistent with hedgehog activation, validating its action as a repressor of the pathway.

Genetically altered mouse models provide experimental evidence linking the hedgehog pathway to human BCC.[9] Targeting SHH or an activated SMO mutant to the epidermis and hair follicles upregulates Shh target genes and produces basal cell–like proliferations in newborn mouse skin. Overexpression of SHH in human keratinocytes followed by grafting onto severe combined immunodeficiency (SCID) mice produced BCC-like changes as well. Mouse models in which *Ptch* gene function has been disrupted develop microscopic hair follicle–derived proliferations, with the appearance of a variety of macroscopic skin tumors, including BCCs, following exposure to ionizing or UV radiation.[10] Mice with skin targeted overexpression of human GLI1 or mouse Gli2 develop multiple BCCs or other tumors arising from hair follicles. The Hh pathway is regulated through primary cilia, present on most mammalian cells, which acts as an organizing structure for signaling and is essential for proteolytic processing of Gli proteins to activator and repressor forms. Inactivation of Kif3a, an essential component of the anterograde intraflagellar transport motor, blocks formation of BCC in mice expressing the activated form of SMO, but accelerates tumor formation driven by activated Gli2. Thus cilia play a dual role in both activating and inhibiting Shh signaling and tumorigenesis.

Taken together, these findings strongly support the concept that deregulated Shh signaling is central for BCC development. When Gli2 is targeted to mouse epidermis and hair follicles conditionally, BCCs develop from overexpression and regress when expression is discontinued. On reexpression of Gli2 in this model, tumors reemerge from a small residual population of precursor cells. What remains unclear is the identification of the hedgehog target genes

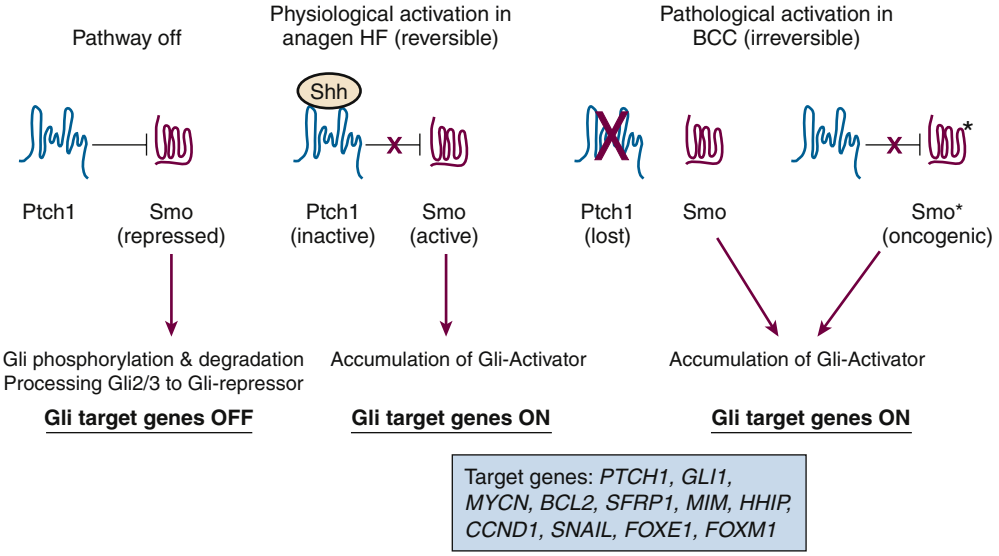


Figure 41-1 Simplified model depicting hedgehog signaling in skin physiology and BCC pathogenesis. The major site of action for sonic hedgehog (SHH) is the hair follicle (HF). In resting hair follicles, SHH is not expressed, and PTCH1 dampens SMO activity. Under these conditions, Gli transcription factors are unstable and degraded. In addition, posttranslational processing of GLI2 and GLI3 processing yields a transcriptional repressor that suppresses SHH target gene expression. When SHH expression increases during HF growth (anagen), PTCH1 is inactivated, SMO is phosphorylated by casein kinase 1 and protein kinase A, and active SMO upregulates Gli proteins by posttranslational mechanisms, leading to accumulation of Gli transcriptional activators. One target of Gli transcription is PTCH1, which serves as a negative feedback on this reversible pathway. In BCC initiation, PTCH1 is genetically deleted (or SMO is mutationally activated to SMO*), and the pathway is irreversibly activated. Gli2 appears to be the major driving factor to upregulate a number of effectors responsible for the neoplastic phenotype, including GLI1, MYCN, BCL2, SFRP1, MIM, HHIP, CCND1, SNAIL, FOXE1, and FOXM1.

downstream from Gli that are essential for BCC formation. A number of candidates have been reported, including several cyclins, E2f1, N-Myc, Pdgfr, Bcl2, BEG4, FOXM1, and FOXE1, Mim, Hhip, Snail, Ptch1, and Gli1 (Figure 41-1), but definitive studies on these genes are lacking. In both human and mouse BCC the Wnt pathway appears dysregulated due to elevated expression of β-catenin, and blockade of Wnt signaling in mice blocks hedgehog-driven tumor growth. Crosstalk between hedgehog and EGFR signaling is synergistic for BCC induction in animal models, suggesting that EGFR inhibitors could have a therapeutic role in the clinic. Small-molecule inhibitors of SMO have been developed based on the natural product cyclopamine, which causes BCC regression in animal models. In recent Phase III clinical trials, the SMO inhibitor vismodegib has caused significant tumor regression in patients with advanced sporadic BCC[11] and regression of preexisting lesions and prevention of new lesions in Gorlin syndrome patients.[12] Vismodegib was approved by the U.S. Food and Drug Administration (FDA) in January 2012 for adult patients with recurrent locally advanced or metastatic BCCs or who are not candidates for surgery or radiation. Other inhibitors are likely to follow, although target-based side effects may be an inherent limitation for long-term treatment. Because the Hedgehog pathway is aberrantly activated in malignancies arising in several internal organs,[1] these inhibitors are likely to have a significant impact on the treatment of a broad range of human neoplasms.

Cutaneous Squamous Cell Carcinoma

Cutaneous SCC frequently presents as a firm, pink papule or nodule, with a conspicuous hyperkeratotic surface. Although SCCs represent only about 20% of nonmelanoma skin cancers, they are invasive and occasionally metastasize (1% to 2%). SCC is more frequent with higher cumulative sunlight exposure and as cancers associated with specific occupational exposures (coke oven and petroleum oil workers). There is roughly a 25- to 200-fold increase in SCC incidence in immunosuppressed organ transplant recipients, with a reversal of the BCC-to-SCC ratio; these tumors are more aggressive, occur in multiple locations within one patient, and are associated with increased morbidity and mortality. Conversely, a drug that activates local innate and adaptive immune responses through TLR-7 (imiquimod (1-(2-methylpropyl)-1*H*-imidazo[4,5-C]quinolin-4-amine) is highly effective in treating BCC, SCC, and its precursor lesion, actinic keratosis (AK).[13] Cutaneous SCC is usually preceded by a benign hyperproliferative-hyperkeratotic AK (Figure 41-2, *A*). These are sunlight-induced clonal lesions that frequently harbor *p53* mutations, particularly at codon 278 or other codons of the DNA binding domain of p53 that contain dipyrimidine sites. AKs often exhibit chromosomal changes, particularly loss of heterozygosity (LOH) at 3p, 13q, 17p, 17q, 9p, and 9q. Such changes are less frequent in SCC, clouding the direct relation of AK to SCC.

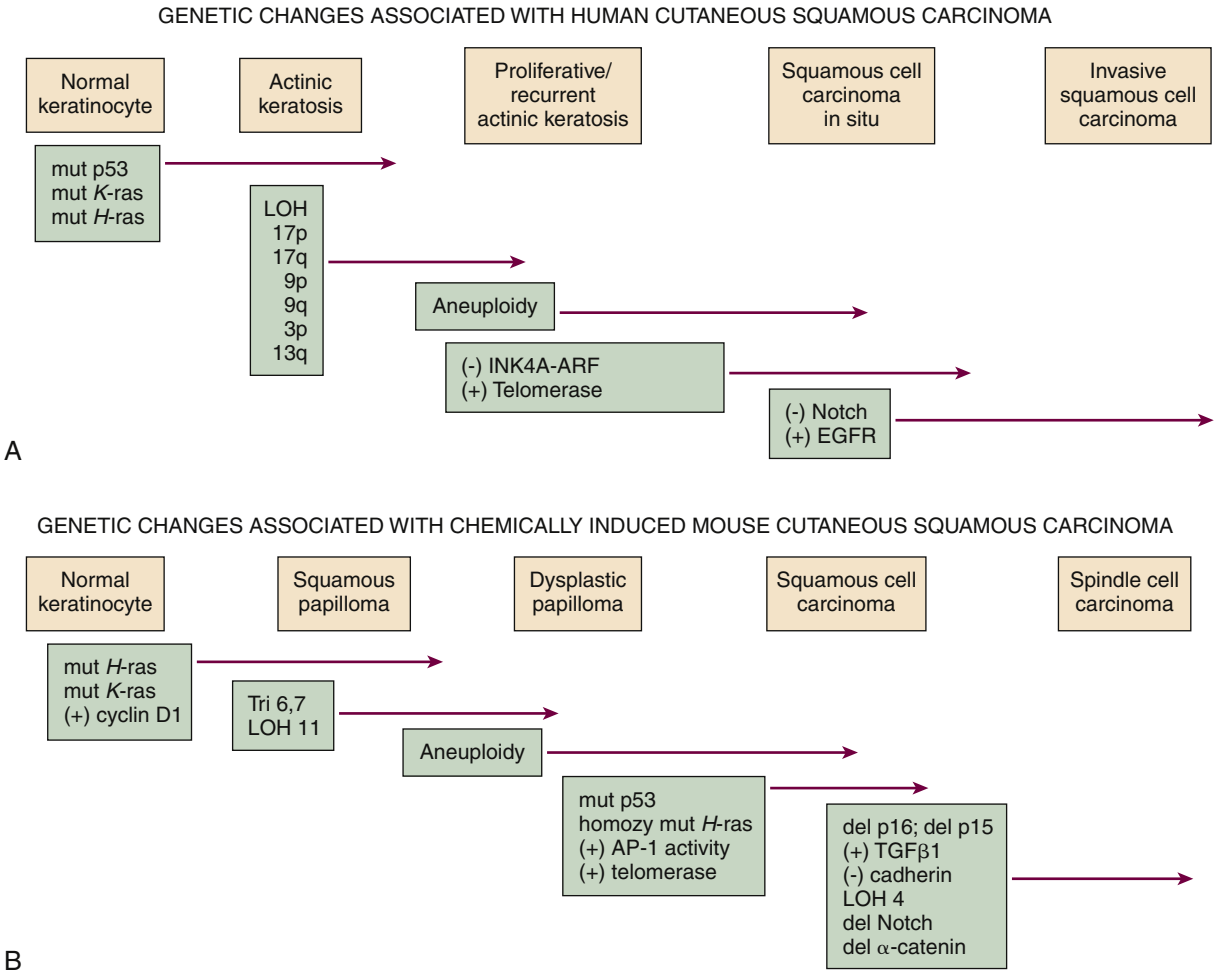


Figure 41-2 Genetic changes associated with (A) human cutaneous squamous carcinoma and (B) experimental mouse skin squamous carcinoma. The multistage evolution of invasive squamous cell cancer is depicted schematically with frequently associated genetic changes detailed here. Although many common pathways exist in the two species, current understanding places p53 mutations early in human SCC development and ultraviolet light–induced mouse skin SCC (not shown), but p53 mutations occur late in chemically induced mouse skin SCC associated with malignant conversion. Increased activity of telomerase (deletion of inhibitor) or EGFR tyrosine kinase (gene amplification) and decreased signaling in the Notch pathway (mutation) may also result from epigenetic changes, as in the case of upregulation of EGFR ligand expression in mouse SCC development. *This figure is modified from Dlugosz A, Merlino G, Yuspa SH. Progress in cutaneous cancer research.* J Invest Dermatol Symp Proc *7:17-26, 2002, with permission of the* Journal of Investigative Dermatology.

However, the frequency of evolution of AK to SCC is very low (0.1% to 10%), suggesting there may be a high-risk AK group with relevant genetic changes not yet documented. Activating mutations in the K-*RAS* or Ha-*RAS* gene are detected in approximately 10% of AK and SCC, and the RAS pathway is activated by non-mutational mechanisms in a much larger fraction of SCCs. Inactivating mutations or epigenetic silencing of p16*(INK4a)* and activation of telomerase are other pathways associated with SCC development. Constitutive activation of the EGF receptor (EGFR) by amplification or increased expression of ligands with the formation of an autocrine loop is a frequent finding in SCC. Gene expression arrays have revealed several other genes whose expression is characteristic of SCC, but experimental validation of a causal relation remains to be determined.

Constitutive activation of NFκB signaling is common in SCC,[14,15] and this is associated with upregulation of specific NFκB target genes associated with altered proliferation, invasion, angiogenesis, and inflammation. NFκB hyperactivation is a known inducer of cylindromatous skin tumors, because homozygous deletions of the CYLD gene, encoding a deubiquitinase that negatively regulates the NFκB pathway through targets such as TRAF2 and BCL-3, has been identified as the basis for familial cylindromatosis (see Table 41-1). Homozygous deletion of CYLD in mice increases susceptibility to squamous skin tumors. However, studies with genetically modified human keratinocytes grafted to nude mice suggest that inhibition of NFκB together with activation of the *RAS* oncogene is sufficient to convert normal keratinocytes into SCC, and a similar result has been obtained in the skin of transgenic mice. This controversy is unsettled at this time.

To prove causal relations among the various associations made by studying human SCC, model systems using

human and mouse keratinocytes in culture and animal models in vivo have been developed. The classical carcinogenesis model to induce SCC on mouse skin involves a limited application of a mutagenic agent such as a chemical carcinogen or UV light that "initiates" the cancer process in a subset of cells, followed by repeated applications of a nonmutagenic agent such as a phorbol ester that provides a microenvironment favorable for the clonal outgrowth of the initiated population. Tumor development proceeds through predictable stages, with the early emergence of benign squamous papillomas, the murine equivalent of AK, some of which progress to invasive SCC, mimicking human SCC with a low metastatic rate (see Figure 41-2, *B*). In some cases SCC will evolve into a more aggressive spindle cell cancer. This model has illuminated the genetic and biochemical changes that are permissive for SCC development under controlled experimental conditions. Heterozygous activating *Ras* gene mutations are sufficient to "initiate" the target cells and produce squamous papillomas, and this is coupled to constitutive activation of the EGFR through overexpression of EGFR ligands (see Figure 41-2, *B*). Papillomas form spontaneously in transgenic mice overexpressing ErbB2 and TGFα in epidermis, and papilloma formation is reduced in mice where EGFR or SOS is ablated. Furthermore, tumor formation is completely inhibited in mice lacking the *Stat3* gene, a downstream target of the EGFR. The clinical relevance of this pathway is highlighted by the effectiveness of anti-EGFR drugs in head and neck SCC patients. The relevance of the RAS pathway in human skin cancer is emphasized by the development of multiple cutaneous SCC and keratoacanthoma expressing *Ha-RAS* mutations in melanoma patients treated with the BRAF V600E inhibitor vemurafenib.[16] This drug also enhanced chemical carcinogenesis on mouse skin through stimulation of MAPK activity. Inactivation of PKCδ is also essential for papilloma development in mouse skin. PKCδ is inactivated by c-Src–mediated tyrosine phosphorylation in initiated mouse keratinocytes, whereas in human skin tumors the expression of PKCδ transcripts is greatly reduced. Transgenic mice overexpressing PKCδ in the skin do not form skin tumors. TGFβ plays a dual role in experimental SCC development, suppressing premalignant progression to SCC while enhancing phenotypic progression from SCC to a spindle cell phenotype.[17] However, topical inhibitors of the TGFβ type I receptor suppress papilloma formation, suggesting that TGFβ signaling is important in tumor outgrowth. Inactivation of p53 enhances malignant conversion of papillomas to SCC in chemical carcinogenesis but is an earlier event in UV light–induced skin carcinogenesis in both mice and humans. Members of the AP-1 transcription factor family also play a dual role in experimental skin tumor development, where c-Jun is essential for papilloma and c-Fos is essential for SCC development. Inhibition of AP-1 activity after the formation of benign papillomas prevents malignant conversion.

In contrast to murine models for BCC, the development of techniques to genetically alter mice greatly expanded the array of genes and pathways that influence SCC development on the skin (Table 41-2), indicating a much more complex pathogenesis. Pathways and genes that can influence SCC development and progression to spindle cell tumors in mice include cyclin D1, ornithine decarboxylase, p16(ink4A), p15(ink4A), p63, E-cadherin, and TGFβ1, and these are frequently altered in human SCC. In several model systems, genetic alterations have resulted in the spontaneous development of SCC in the absence of a precursor lesion. For example, mice ablated for *Notch* or *β-Catenin* rapidly form SCCs even in the absence of carcinogenic exposure, and ablation of *Notch* in epidermis promotes tumor formation by creating a barrier defect and promoting inflammation.[18] In fact, loss of function *Notch* mutations is frequently found in cutaneous SCC.[19] Furthermore, clinical trials of γ-secretase inhibitors for the treatment of Alzheimer's disease have been discontinued because of emergence of NMSC.[20] γ-Secretase is required for the activation of intracellular Notch action.

Defining the Cell of Origin for Cutaneous Cancers

Genetic profiling of both SCC and BCC indicate a monoclonal origin. However, the multiplicity of epithelial tumor phenotypes in skin (see Table 41-1) and the divergent phenotypes within each suggest that either multiple target cells exist within the complex compartmentalization of the integument or that specific genetic lesions or tumor microenvironments acting on a multipotential target cell determine tumor phenotype and malignant potential.[21,22] Both human and murine skin contains several types of stem cells within different locations in the epidermis and hair follicle that maintain discrete epidermal compartments, with stem cells within the bulge region of the hair follicle the best characterized.[22] Gene expression analysis of isolated murine hair follicle stem cells and analysis of genetically altered mice has revealed specific gene expression patterns and signaling pathways that define stemness, and Lgr6+ cells residing above the follicle bulge may mark stem cells capable of generating all three self-renewing compartments of the epidermis.[23]

Lineage tracing studies in mice and targeted activation of Hh signaling in different cells of the hair follicle have revealed that only cells originating in the interfollicular epidermis and upper infundibulum are capable of generating BCC in the absence of other factors, such as wounding hair growth cycle, irradiation, or supraphysiological expression of

Table 41-2 Genetically Modified Mouse Models for Cutaneous Squamous Cell Cancer

Modification	Enhancers	Comments
Tg.AC (ζ globin-v-ras^{Ha})	Promoters, drugs	Enhancers upregulate transgene
Tg.AC	UVB	p53 mutations are absent
K1-ras, K10-ras	Promoters	Predominantly papillomas
ΔK5-ras	None	Papillomas, KA, SCC
K6-ras	Promoters	SCC
K1-TGFα, K14-TGFα, MT-TGFα	Promoters	Predominantly papillomas that regress
Inv-c-MycER	None	Papilloma
K1-v-fos	Promoters	Papilloma
K5-E2F1	p53 deficiency	Papilloma, SCC, BCC
K5-Igf1	Promoters	Papilloma, SCC
K5-ErbB2	Promoters	Papilloma, SCC
K5-SOS-F	None	Tumors inhibited by Egfr deficiency
K14-HPV16	FVB/N mouse strain	Tumors inhibited by difluoromethylornithine
K6-ODC	DMBA	SCC, K-ras mutations
XP mutant models (A, C, D)	Initiation/promotion/UVR	Enhanced sensitivity
Egfr null mutant	v-*ras*^{Ha}	Reduced tumor size
p53 null mutant	DMBA/TPA	Enhanced malignant conversion
p21^{waf1} null mutant	DMBA/TPA	Enhanced papilloma formation
c-fos null mutant	Cross with Tg.AC	Papilloma but no SCC
K14-PKCε	DMBA/TPA	Enhanced SCC, metastases
K14-PKCδ	DMBA/TPA	Reduced papilloma development
K5-src	None or DMBA/TPA	Enhanced spontaneous or induced SCC
K5-IκB mutant	None	Spontaneous SCC/NFκB inhibition
Notch null	None	Spontaneous SCC/nuclear β-catenin
α-catenin null	None	Spontaneous SCC/NFκB activation
Cyld null	None or DMBA/TPA	Enhanced papilloma/NFκB activation

pathway components. Targeting high levels of oncogenic Ras to basal and stem cell keratinocytes gives rise to squamous tumors with a high risk for malignant conversion, whereas targeting suprabasal keratinocytes produces only benign tumors. Lineage tracing with genetically altered mice shows that keratin 15 expressing cells of the hair follicle bulge region contribute to chemically induced squamous papillomas.[24] However, expression of physiological levels of oncogenic Ras in hair follicle bulge stem cells, hair germ, and outer root sheath or interfollicular keratinocytes only gives rise to benign papilloma, but SCC can develop in all cells when combined with p53 deletion. More committed hair matrix keratinocytes were unable to form tumors with Ras or Ras + p53 loss.[25,26] Although the cell of origin has not been identified for human cutaneous SCC, tumor-initiating or cancer stem cells (CSCs) have been isolated from cutaneous SCC based on expression of CD133 (prominin), which can regenerate tumors with similar histology and grade as the original in serial xenotransplants on immunocompromised mice.[27] In murine cutaneous SCC, 2 CSC populations have been identified differing in the expression of the hair follicle bulge marker CD34, and responsiveness to TGFβ1 signaling and FAK-mediated integrin signaling. Other studies have demonstrated dependence of CSC on β-catenin signaling and autocrine VEGF responses requiring the neuropilin receptor. Together these mouse models show that SCC can arise from multiple cells within the hair follicle and interfollicular epidermis, that distinct CSC populations exist within the same tumor, and that they have gene expression patterns distinct from normal hair follicle stem cells and require specific

microenvironmental stimuli and gene expression pathways to maintain the CSC phenotype.

Importance of the Microenvironment in Cutaneous Cancer

Although much cutaneous cancer research has focused on cell-autonomous alterations in keratinocytes that contribute to cancer development, it is now clear that alterations in the cutaneous tissue environment are also critical. Changes in integrin distribution and expression occur during progression of human and chemically induced mouse SCC, and targeted changes in specific integrin complex expression can enhance or suppress malignant conversion. Similarly, mutations in the anchoring molecule collagen VII that block the interaction of collagen VII with laminin 5 predispose dystrophic epidermolysis bullosa to SCC.

Changes in the cutaneous immune microenvironment are also critical for tumor development. Inflammation and immunosuppression caused by UV irradiation are intimately linked to UV-induced skin cancer. Similarly, inflammation within the tumor microenvironment is associated with the progression of AK to SCC. Genetically altered mice lacking skin-resident γδ T cells have increased frequency of chemically induced papillomas and malignant conversion, suggesting that this resident T cell population is important in antitumor immunosurveillance. Surprisingly, Langerhans cells, the other epidermal resident immune cell, mediate chemical carcinogenesis through effects on carcinogen metabolism allowing DNA damage and mutation in keratinocytes. In contrast, mice with a TCRβ deletion (lacking all αβ T cells) have a significantly reduced carcinoma yield, and this may be due to a subset of tumor-promoting $CD8^+$ T cells.[28,29] Humoral immunity and B cells also play a tumor-promoting role in skin carcinogenesis through cutaneous deposition of IgG that enhances recruitment of proinflammatory myeloid cells. Factors that mediate inflammation such as prostaglandins, TNFα, and IFNγ enhance experimental cutaneous carcinogenesis. Likewise, chronic inflammatory skin conditions such as discoid lupus erythematosus, dystrophic epidermolysis bullosa, and chronic wounds are associated with increased susceptibility to human skin cancer.

Perspective

Advances in understanding the molecular basis of cutaneous cancer have reinforced the paradigm that particular genetic and epigenetic changes and the pathways they regulate contribute to skin cancer formation in a stage-specific manner. What benefit may come from these current insights? This is most clear in the case of BCC, where the molecular mechanism of pathogenesis is so precisely defined (perhaps better than in any other human cancer) that curative therapeutic targets are identified, and precise animal models have been developed for testing new therapeutics. Currently cyclopamine and derivatives with better therapeutic index are in clinical trials to block SMO, with remarkable results in advanced patients,[11] and tazarotene, a retinoid used successfully to treat BCC lesions in mouse models, is an inhibitor of GLI function.[30] Although BCC is generally not life threatening, the high frequency of these lesions on exposed skin favors a medical rather than the traditional surgical approach, an advance that is being achieved by translation of basic research. Recent advances in tools for large-scale expression and genomic analysis have been applied to SCC lesions and their precursor AK. Animal models and human tissue analyses have suggested that premalignant precursor lesions vary in risk for progression, and it is anticipated that molecular profiling will reveal markers to identify high-risk lesions for closer clinical scrutiny. Similarly, profiling of SCC will undoubtedly reveal signature markers associated with lesions at risk for metastatic spread. Currently two molecular therapeutic targets derived from basic research on SCC pathogenesis show promise for medical therapy of SCC. Inhibitors of the EGFR, in clinical use for several internal malignancies, show promise in animal models for the prevention of UV-induced mouse skin SCC. Ingenol-3-angelate (Picato), recently approved for treatment of AK, BCC, and SCC in situ, targets protein kinase C to induce an innate immune response and damage tumor vasculature.[31] Stimulation of innate immunity to destroy skin tumors is another paradigm for cancer therapy, first introduced into the clinic with the drug imiquimod (Aldara) that targets Toll-like receptor-7. Other targets identified from experimental studies that offer therapeutic potential are telomerase, TGFβ, Notch, and p53, because drugs targeting these molecules are in clinical trials for treating a number of epithelial cancers.

A developing concept anticipated from molecular analyses is that common gene or protein expression profiles would reveal similar pathogenic mechanisms for skin SCC and squamous tumors of the lung, head, and neck and other sites that pose a threat to life. Similarly, data already exist indicating that the hedgehog pathway is involved in internal malignancies such as pancreatic cancer. If such mechanisms are shared, then new drugs could be tested on skin tumors for therapeutic efficacy. The high frequency of skin cancers, their superficial location, and the capacity for topical testing suggest that the skin provides an excellent surrogate site for evaluating drug development for a variety of internal tumor sites. The skin is also a site that often predicts the presence of

internal tumors with such lesions as acanthosis nigricans, dermatomyositis, paraneoplastic pemphigus, and other dermatoses. Little is known of the pathogenesis of these premonitory lesions, but undoubtedly such knowledge would reveal important aspects of the host response to cancer. Thus, progress in skin cancer research will continue to provide important translational opportunities not just for these very prevalent lesions, but for the advancement of cancer treatment in general.

Acknowledgments

Because of the breadth of this review, we apologize for the unavoidable exclusion of references to work done by many outstanding investigators working in these areas. We acknowledge Professor Andrzej Dlugosz for advice on BCC pathogenesis.

Key References

1. Teglund S, Toftgard R. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta*. 1805;181:2010.
2. DiGiovanna JJ, Kraemer KH. Shining a light on xeroderma pigmentosum. *J Invest Dermatol*. 2012;132:785.
3. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science*. 1996;272:1668.
4. Han J, Qureshi AA, Nan H, et al. A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer Res*. 2011;71:1533.
5. Kosiniak-Kamysz A, Pospiech E, Wojas-Pelc A, et al. Potential association of single nucleotide polymorphisms in pigmentation genes with the development of basal cell carcinoma. *J Dermatol*. 2012.
6. Nan H, Xu M, Kraft P, et al. Genome-wide association study identifies novel alleles associated with risk of cutaneous basal cell carcinoma and squamous cell carcinoma. *Hum Mol Genet*. 2011;20:3718.
7. Teh MT, Blaydon D, Chaplin T, et al. Genomewide single nucleotide polymorphism microarray mapping in basal cell carcinomas unveils uniparental disomy as a key somatic event. *Cancer Res*. 2005;65:8597.
8. Mill P, Mo R, Fu H, et al. Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev*. 2003;17:282.
9. Kasper M, Jaks V, Are A, et al. Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. *Proc Natl Acad Sci U S A*. 2011;108:4099.
10. So PL, Lee K, Hebert J, et al. Topical tazarotene chemoprevention reduces Basal cell carcinoma number and size in Ptch1+/– mice exposed to ultraviolet or ionizing radiation. *Cancer Res*. 2004;64:4385.
11. Sekulic A, Migden MR, Oro AE, et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *N Engl J Med*. 2012;366:2171.
12. Tang JY, kay-Wiggan JM, Aszterbaum M, et al. Inhibiting the hedgehog pathway in patients with the basal-cell nevus syndrome. *N Engl J Med*. 2012;366:2180.
13. Eklind J, Tartler U, Maschke J, et al. Imiquimod to treat different cancers of the epidermis. *Dermatol Surg*. 2003;29:890.
14. Kobielak A, Fuchs E. Links between alpha-catenin, NF-kappaB, and squamous cell carcinoma in skin. *Proc Natl Acad Sci U S A*. 2006;103:2322.
15. Loercher A, Lee TL, Ricker JL, et al. Nuclear factor-kappaB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res*. 2004;64:6511.
16. Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med*. 2012;366:207.
17. Glick AB. TGFbeta1, Back to the Future: Revisiting its Role as a Transforming Growth Factor. *Cancer Biol Ther*. 2004;3:276.
18. Demehri S, Turkoz A, Kopan R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. *Cancer Cell*. 2009;16:55.
19. Wang NJ, Sanborn Z, Arnett KL, et al. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2011;108:17761.
20. Extance A. Alzheimer's failure raises questions about disease-modifying strategies. *Nat Rev Drug Discov*. 2010;9:749.
21. Singh A, Park H, Kangsamaksin T, et al. Keratinocyte Stem Cells and the Targets for Nonmelanoma Skin Cancer(dagger). *Photochem Photobiol*. 2012.
22. Beck B, Blanpain C. Mechanisms regulating epidermal stem cells. *EMBO J*. 2012;31:2067.
23. Snippert HJ, Haegebarth A, Kasper M, et al. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. *Science*. 2010;327:1385.
24. Li S,Park H,Trempus CS et al:A keratin 15 containing stem cell population from the hair follicle contributes to squamous papilloma development in the mouse. 2013;10:751-759.
25. Lapouge G, Youssef KK, Vokaer B, et al. Identifying the cellular origin of squamous skin tumors. *Proc Natl Acad Sci U S A*. 2011;108:7431.
26. White AC, Tran K, Khuu J, et al. Defining the origins of Ras/p53-mediated squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2011;108:7425.
27. Patel GK, Yee CL, Terunuma A, et al. Identification and characterization of tumor-initiating cells in human primary cutaneous squamous cell carcinoma. *J Invest Dermatol*. 2012;132:401.
28. Kwong BY, Roberts SJ, Silberzahn T, et al. Molecular Analysis of Tumor-Promoting CD8(+) T Cells in Two-Stage Cutaneous Chemical Carcinogenesis. *J Invest Dermatol*. 2009;130:1726.
29. Roberts SJ, Ng BY, Filler RB, et al. Characterizing tumor-promoting T cells in chemically induced cutaneous carcinogenesis. *Proc Natl Acad Sci U S A*. 2007;104:6770.
30. So PL, Fujimoto MA, Epstein Jr EH. Pharmacologic retinoid signaling and physiologic retinoic acid receptor signaling inhibit basal cell carcinoma tumorigenesis. *Mol Cancer Ther*. 2008;7:1275.
31. Li L, Shukla S, Lee A, et al. The skin cancer chemotherapeutic agent ingenol-3-angelate (PEP005) is a substrate for the epidermal multidrug transporter (ABCB1) and targets tumor vasculature. *Cancer Res*. 2010;70:4509.

This page intentionally left blank

References

1. Teglund S, Toftgard R. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta*. 1805;181:2010.
2. DiGiovanna JJ, Kraemer KH. Shining a light on xeroderma pigmentosum. *J Invest Dermatol*. 2012;132:785.
3. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science*. 1996;272:1668.
4. Han J, Qureshi AA, Nan H, et al. A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer Res*. 2011;71:1533.
5. Kosiniak-Kamysz A, Pospiech E, Wojas-Pelc A, et al. Potential association of single nucleotide polymorphisms in pigmentation genes with the development of basal cell carcinoma. *J Dermatol*. 2012.
6. Nan H, Xu M, Kraft P, et al. Genome-wide association study identifies novel alleles associated with risk of cutaneous basal cell carcinoma and squamous cell carcinoma. *Hum Mol Genet*. 2011;20:3718.
7. Teh MT, Blaydon D, Chaplin T, et al. Genomewide single nucleotide polymorphism microarray mapping in basal cell carcinomas unveils uniparental disomy as a key somatic event. *Cancer Res*. 2005;65:8597.
8. Mill P, Mo R, Fu H, et al. Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. *Genes Dev*. 2003;17:282.
9. Kasper M, Jaks V, Are A, et al. Wounding enhances epidermal tumorigenesis by recruiting hair follicle keratinocytes. *Proc Natl Acad Sci U S A*. 2011;108:4099.
10. So PL, Lee K, Hebert J, et al. Topical tazarotene chemoprevention reduces Basal cell carcinoma number and size in Ptch1+/− mice exposed to ultraviolet or ionizing radiation. *Cancer Res*. 2004;64:4385.
11. Sekulic A, Migden MR, Oro AE, et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *N Engl J Med*. 2012;366:2171.
12. Tang JY, kay-Wiggan JM, Aszterbaum M, et al. Inhibiting the hedgehog pathway in patients with the basal-cell nevus syndrome. *N Engl J Med*. 2012;366:2180.
13. Eklind J, Tartler U, Maschke J, et al. Imiquimod to treat different cancers of the epidermis. *Dermatol Surg*. 2003;29:890.
14. Kobielak A, Fuchs E. Links between alpha-catenin, NF-kappaB, and squamous cell carcinoma in skin. *Proc Natl Acad Sci U S A*. 2006;103:2322.
15. Loercher A, Lee TL, Ricker JL, et al. Nuclear factor-kappaB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res*. 2004;64:6511.
16. Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med*. 2012;366:207.
17. Glick AB. TGFbeta1, Back to the Future: Revisiting its Role as a Transforming Growth Factor. *Cancer Biol Ther*. 2004;3:276.
18. Demehri S, Turkoz A, Kopan R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. *Cancer Cell*. 2009;16:55.
19. Wang NJ, Sanborn Z, Arnett KL, et al. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2011;108:17761.
20. Extance A. Alzheimer's failure raises questions about disease-modifying strategies. *Nat Rev Drug Discov*. 2010;9:749.
21. Singh A, Park H, Kangsamaksin T, et al. Keratinocyte Stem Cells and the Targets for Nonmelanoma Skin Cancer(dagger). *Photochem Photobiol*. 2012.
22. Beck B, Blanpain C. Mechanisms regulating epidermal stem cells. *EMBO J*. 2012;31:2067.
23. Snippert HJ, Haegebarth A, Kasper M, et al. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. *Science*. 2010;327:1385.
24. Li S,Park H,Trempus CS et al: A keratin 15 containing stem cell population from the hair follicle contributes to squamous papilloma development in the mouse. 2013;10:751-759.
25. Lapouge G, Youssef KK, Vokaer B, et al. Identifying the cellular origin of squamous skin tumors. *Proc Natl Acad Sci U S A*. 2011;108:7431.
26. White AC, Tran K, Khuu J, et al. Defining the origins of Ras/p53-mediated squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2011;108:7425.
27. Patel GK, Yee CL, Terunuma A, et al. Identification and characterization of tumor-initiating cells in human primary cutaneous squamous cell carcinoma. *J Invest Dermatol*. 2012;132:401.
28. Kwong BY, Roberts SJ, Silberzahn T, et al. Molecular Analysis of Tumor-Promoting CD8(+) T Cells in Two-Stage Cutaneous Chemical Carcinogenesis. *J Invest Dermatol*. 2009;130:1726.
29. Roberts SJ, Ng BY, Filler RB, et al. Characterizing tumor-promoting T cells in chemically induced cutaneous carcinogenesis. *Proc Natl Acad Sci U S A*. 2007;104:6770.
30. So PL, Fujimoto MA, Epstein Jr EH. Pharmacologic retinoid signaling and physiologic retinoic acid receptor signaling inhibit basal cell carcinoma tumorigenesis. *Mol Cancer Ther*. 2008;7:1275.
31. Li L, Shukla S, Lee A, et al. The skin cancer chemotherapeutic agent ingenol-3-angelate (PEP005) is a substrate for the epidermal multidrug transporter (ABCB1) and targets tumor vasculature. *Cancer Res*. 2010;70:4509.

Marc S. Ernstoff

42

Molecular Basis for Treating Cutaneous Molecular

Introduction

The mapping of the human genome, and more recently the unraveling of human cancer–specific genomes, has led to more detailed understanding of carcinogenesis, cancer growth, progression, and metastasis. With this understanding, new treatment paradigms for cancer have been developed, generating novel agents that have been successfully introduced into the clinic.

Like normal tissues, tumor tissue exploits growth-receptor signal pathways for its maintenance and progression. Disruption of critical pathways can lead to cancer cell death, translating into clinical response, palliation of cancer-related symptoms, and improvement of overall survival for cancer patients. On the other hand, mutations in the growth receptor signal transduction pathway can lead to "addiction" of the tumor to this pathway, resulting in uncontrolled growth. Effective blockade of the mutated protein or its downstream signaling events leads to inhibition of cancer growth and cancer cell death.

The story behind the development of the first targeted therapy, imatinib, illustrates the course of the paradigm shift now gripping the cancer therapeutic world. Since 1960 we have known about the Philadelphia chromosome's association with chronic myelogenous leukemia (CML).[1] Yet the translocation between chromosome 9 and 22 was not described until 1973, and over the subsequent years the translocation was linked to the Abelson oncogenic virus and its human counterpart.[2-6] In the late 1990s Nicholas Lydon and Brian Druker developed imatinib, a compound that blocks BCR-Abl.[7] Because BCR-Abl transcript is constitutively expressed in CML, blocking this path led to remarkable responses in patients with the disease. Imatinib received approval by the U.S. Food and Drug Administration (FDA) for the treatment of CML in 2001, and the paradigm shift in cancer therapeutics took hold.

The implications of this paradigm shift have subsequently led to the identification of many growth-receptor signal transcription kinase–mediated pathways that are either upregulated or mutated and thus targetable for drug development. As experience grows with these new targeted agents, we are discovering the importance of crosstalk with other pathways of cancer destruction, including host-mediated factors attributable to the immune system or to stroma. In this chapter we review the areas of targetable therapy related to melanoma.

Melanoma Background

Melanoma incidence in the U.S. population continues to rise and is associated with a modest increase in mortality rates, primarily in men.[8,9] Nearly 50% of skin melanomas are diagnosed before the age of 55, thus having a significant impact on a population of individuals in the prime of their working lives.[9] Understanding carcinogenesis, predicting clinical course, and developing new treatments is a high priority as melanoma continues to exasperate biomedical scientists and clinicians, with devastating consequences for patients. When diagnosed in its earliest stages, surgical excision can cure many but not all.[10,11] Our current knowledge about the risk for primary melanoma to metastasize uses simple tumor characteristics that can be seen by microscopic evaluation and include the depth of invasion from the surface of the skin, whether the tumor is ulcerated, and the number of mitoses in the vertical growth phase.[11] Unfortunately, even shallow primary melanomas with a good prognosis metastasize and cause death, suggesting that the process of invasion into tissue and spread through the blood or lymphatic systems is much more complex.

Melanoma Pathology

Melanoma is a tumor that arises from melanocytes, the pigment-producing cells of the body (Figure 42-1). Melanocytes arise from the neural crest and primarily populate the skin and eye, although they can be found in various other organs. The overwhelming majority of melanomas arise from the skin and more rarely the uveal tract of the eye. Cutaneous melanocytes are located at the junction between the epidermis and dermis, at the base of the rete ridges. Melanocytes are dendritic-type cells that communicate and provide pigmentation (melanin) to keratinocytes of the skin. They increase pigmentation production in response to UV exposure. Interestingly, melanin pigmentation migrates to the UV-exposed side of the nucleus, providing shielding to DNA-damaging UV light.[12]

Melanocytes will transform into nevus cells that are able to form theques or nests at the junction of the skin layers (see Figure 42-1). These pigmented lesions are called *junctional nevi* and are flat, homogeneous, brown or tan macules that are well defined. Nevic cells have the ability to invade or migrate into the dermis, forming a macular-papular lesion or compound nevus or a dermal nevus if all the nests are in the dermis.[13] Dermal nevi tend to lose their pigmentation, frequently presenting as a flesh-colored papule. Nevi are acquired lesions and slowly change over time. Newborn infants rarely have nevi, and older individuals, although they have numerous pigmented skin lesions, also have few true nevi. A variation of nevi termed *atypical nevi* or *dysplastic nevi* has been recognized and has a different growth pattern, variable amounts of cytological atypia, and a host response identified by lamellar fibroplasia, neovascularization, and lymphocytic infiltration.[14] The number of nevi and atypical nevi are known to be individual risk factors for the development of melanomas.[15] Pathologic series can identify a preexisting nevus in up to 50% of melanomas, suggesting that a precursor lesion exists.[16]

Melanoma typically appears on the sun-exposed areas of the skin, and the amount of sunlight exposure over life is a risk factor. Melanomas arising in sun-damaged skin differ in their molecular pathway expression compared to melanomas from non–sun-damaged skin.[17]

Cutaneous melanomas appear in four major distinct clinical and histologic variants: nodular, superficial spreading, lentigo maligna, and acral lentiginous melanomas. Although molecular pathways may differ between these variants, the risk for metastasis remains linked to the depth of invasion into the skin. Superficial spreading melanoma (SSM) is the most common form of the disease and is typically present for years on the skin. It has the hallmark of the classic ABCDE characteristics popularly known as *a*symmetry, irregular *b*orders, variegated *c*olor, larger *d*iameter (greater than 6 mm), and *e*volving over time.[18] SSM has radial growth and nodular or vertical growth. Nodular melanoma has a vertical growth phase without any evidence of horizontal growth. Nodular melanomas usually appear rapidly and invade the dermis quickly. Acral lentiginous melanoma is seen on the palms, soles, and nail beds and is the most frequent melanoma in dark-skinned people. Lentigo maligna melanoma is the invasive form of lentigo maligna (LM), which is a melanoma in situ arising in sun-damaged skin. LMs are typically noted on the skin for many years.

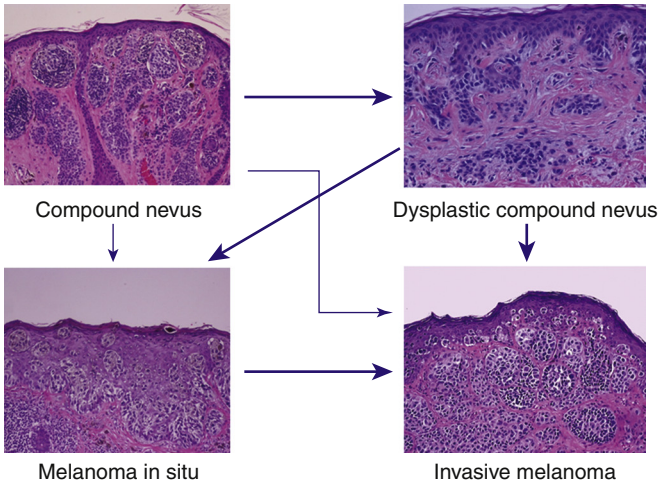


Figure 42-1 Development of melanoma Although melanomas can develop from melanocytes at the epidermal-dermal junction directly or in a preexisting nevus, an orderly progression from melanocyte to neoplasm (benign nevus) to dysplasia to noninvasive (in situ) cancer to overt invasive malignancy can also be seen in clinical material. This figure shows the histopathologic features of a compound nevus, dysplastic nevus, melanoma in situ, and invasive melanoma.

Melanoma Therapy and Molecular Targets

Historically, melanoma has been one of the most unresponsive cancers to traditional chemotherapy approaches. Dacarbazine, an agent that methylates the 7-position of guanine on DNA, crosslinks DNA strands, leading to inhibition of DNA, RNA, and protein synthesis. Treatment with this agent demonstrates an 8% objective response rate in metastatic melanoma.[19] Studies in the 1980s suggested that multiagent chemotherapy could enhance the activity of dacarbazine in patients with metastatic melanoma, but a subsequent randomized study failed to show improvement in overall survival with multiagent chemotherapy compared to dacarbazine alone.[19] The first modern-era immune therapies for melanoma, interferon alpha and interleukin-2, introduced in the 1980s, showed tantalizing results and are still considered part of the therapeutic armamentarium despite recent advances.[20,21]

As molecular pathways related to melanoma carcinogenesis and progression have been discovered, novel targets and agents specific for these targets have also been identified. In addition, the molecular pathways mediating immune response to melanoma may also provide novel strategies for treatment. The most relevant new approaches for melanoma in 2012 target BRAF, c-Kit, and CTLA4.[22-24] We review these approaches first and explore other pathways that will likely be relevant for the future discovery of additional therapeutic agents.

Mapk-Braf and Intersection of Other Pathways

The mitogen-activated protein kinase pathway (MAPK) is an oncogenic pathway that mediates growth and progression (Figure 42-2). Growth factor receptor tyrosine kinases (RTK) associated with RAS initiate this pathway, among others, leading to activation of BRAF, MEK, and ERK in the cytoplasm. Phosphorylated ERK migrates to the nucleus and causes cell proliferation. The three members of the RAS proto-oncogene family include HRAS, NRAS, and KRAS, all with GTPase activity. NRAS is the most frequently mutated gene of this family and is seen in 25% to 35% of melanomas.[25,26] The most frequent mutation in NRAS is arginine (R) substituted for a glutamine (Q) at position 61 (Q61R). Downstream from NRAS is RAF. There are three human isoforms of RAF (ARAF, BRAF, and CRAF), which have different cellular locations and different activation sequences. BRAF has constitutive phosphorylation of the N terminus, which is different than for ARAF and CRAF and thus can be activated directly by RAS.[27] The most common RAF mutation is in BRAF, which has been identified in as many as 66% of melanomas.[28] BRAF mutation is also seen in a high percentage of benign nevi, suggesting that mutation of BRAF is seen early in the transformation of melanocytes at the basal layer of the dermal-epidermal junction into tumor (benign and malignant).[28,29] The highest frequency mutation of BRAF occurs at the 600 position with a substitution of a glutamic acid (E) for valine (V) (BRAF[V600E]). This mutation constitutively maintains activation of a downstream event on MEK and ERK, a process referred to as *addiction to an oncogenic pathway*.

Cancers addicted to an oncogenic pathway are susceptible to inhibition by blockade of that pathway. RAS activation through a number of RTKs, including epidermal (EGFR), platelet-derived (PDGFR), and vascular endothelial (VEGFR) growth factor receptors, mediate not only tumor growth but also tumor-associated angiogenesis. Sorafenib (Bay 43-9006) is a biaryl urea developed as a RAF inhibitor and was the first of its kind to enter clinical trials. It had demonstrable activity against RAF targets, including the mutant BRAF (BRAF[V600E], BRAF[V600K], and BRAF[V600M]).[30] In a Phase II melanoma study, 19% of 37 patients had stable disease, but there was no relationship of response to BRAF(V600E) status.[31] Subsequent studies with combined chemotherapy and sorafenib failed to demonstrate significant activity.[32,33]

Using a structure-guided approach to drug discovery, PLX4720 (vemurafenib) was developed as a selective BRAF(V600E) inhibitor.[34,35] PLX4720 binds to the ATP binding site on active BRAF but not BRAF in its inactive conformation. Inhibitors that bind in and around the region occupied by the adenine ring of ATP are referred to as type I inhibitors, whereas sorafenib is a type II inhibitor that instead occupies a hydrophobic site directly adjacent to the ATP pocket.[35] The first Phase I study of PLX4720 or vemurafenib demonstrated significant activity in metastatic melanoma patients, and subsequent randomized studies confirmed the activity, which led to FDA approval for its use in the treatment of metastatic melanoma.[22,36]

In the Phase III study, 675 eligible patients with metastatic melanoma stage IIIC, M1a, M1b, and M1c were randomized in a 1:1 ratio to receive oral vemurafenib (960 mg twice daily) or dacarbazine (1000 mg/m^2 IV every 3 weeks). At the time of analysis, the hazard ratio for death was 0.37 (95% confidence interval 0.26 to 0.55; $P < .0001$) and for progression-free survival 0.26 (95% confidence interval 0.20 to 0.33; $P < .0001$) favoring the vemurafenib treatment. Two complete responses and 104 partial responses were reported in the 219 evaluable subjects who received vemurafenib. Although the toxicity profile for vemurafenib was mild, with cutaneous toxicity being most significant, dose interruption and modification were required in 38% of subjects. The paradoxical activation of CRAF leads to the development of squamous cell cancers and keratoacanthomas.[37] Subsequent studies have demonstrated a median duration of response to BRAF blockade of approximately 7 months.[38] Other BRAF-targeted molecules are under development.[39]

Although targeting mutant BRAF has had significant impact on the therapeutic paradigm, development of resistance continues to be a barrier for prolonged responses in the majority of patients. Development of resistance to BRAF-targeted agents is complex and multifaceted but appears to be driven by reactivation of the MAPK pathway. One mechanism that has been identified is a 61-kDa splice variant of BRAF(V600E) that lacks the RAS-binding domain at exon 4-8 (p61BRAF[V600E]).[40] p61-BRAF(V600E) shows enhanced dimerization and is resistant to known RAF inhibitors. Increased expression of serine/threonine kinases on BRAF, CRAF, or COT1 may also be involved.[40-42] The activating mutations Q61K/R on N-RAS and C212S on MEK1 have also been implicated in BRAF inhibitor resistance by phosphorylation of ERK.[43,44]

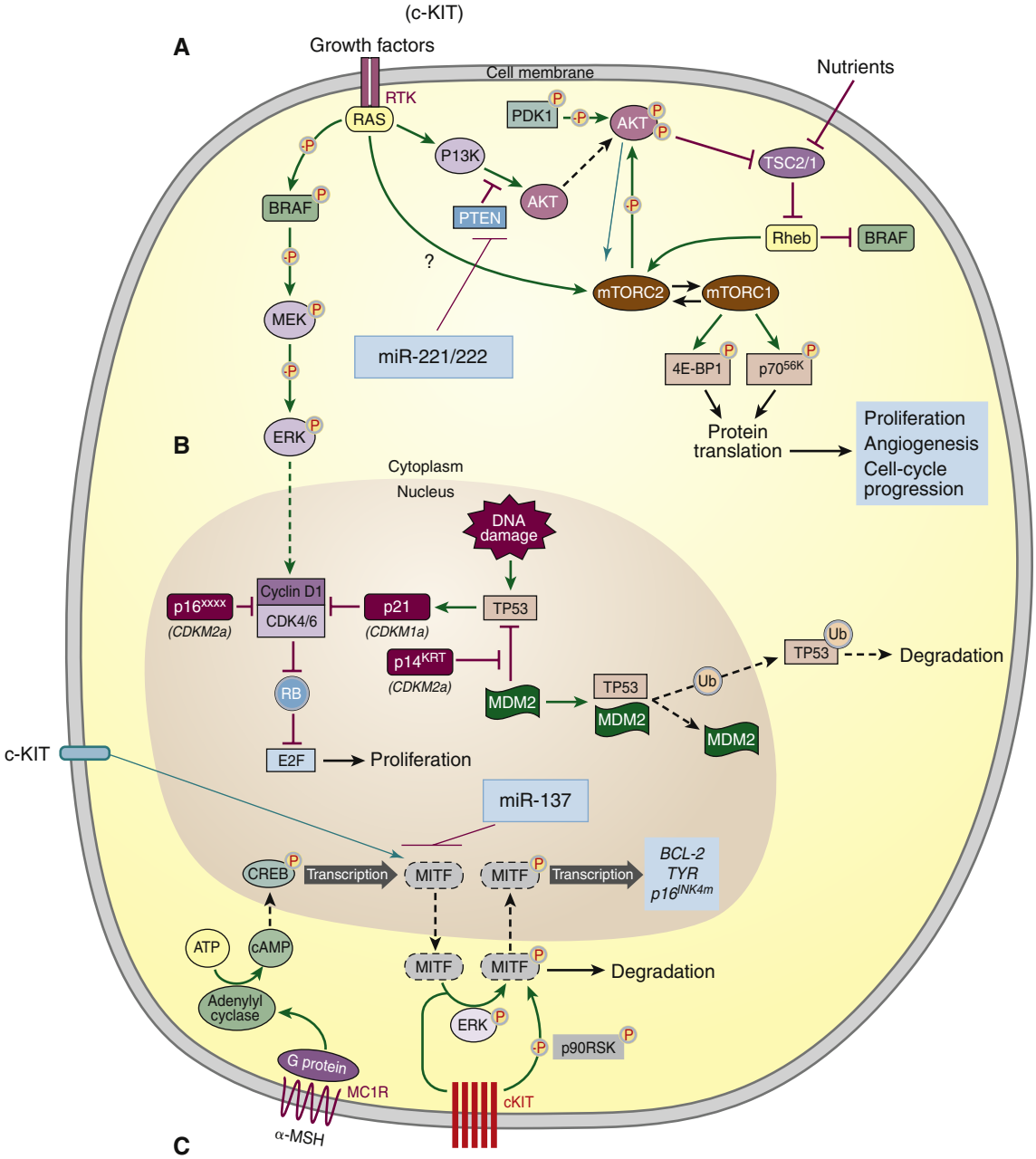


Figure 42-2 (A, B) RTK-RAS activation of MAPK and AKT pathways. *Arrows* represent activation pathways and T (⊥) represents inhibition. In the AKT pathway, PTEN (phosphatase and tensin homologue) dephosphorylates PIP_2 (phosphatidylinositol bisphosphate) and acts in an inhibitory manner. miRNA 221/222 can modulate this pathway by inhibiting PTEN. Activation of AKT is associated with a conformational change and translocates close to the cell membrane where it is phosphorylated by PDK1 (3-phosphoinositide-dependent kinase 1) and then can inhibit TSC2 (tuberous sclerosis protein 2) and activate mTORC2 (mammalian target of rapamycin C2). The MAPK pathway is triggered through RAS activation, ultimately leading to phosphorylated ERK translocating to the nucleus, leading in turn to cellular proliferation through cyclin D1 complex. DNA damage activates TP53 (tumor protein 53), which in turn activates CDKN1a (cyclin-dependent kinase inhibitor 1a), suppresses cyclin D1-$CDK_4/_6$ complex, and inhibits transcription via RB (retinoblastoma). MDM2 (mouse double minute-2) inhibits TP53 by binding it and making available for degradation through the ubiquitin pathway. p14(ARF) sequesters MDM2 and thus inhibits TP53 degradation. **(C)** α-MSH (melanocyte-stimulating hormone) binds to MC1R (melanocortin-1 receptor), stimulating a cascade that transcribes MITF (microphthalmia transcription factor) gene. miR-137 can modulate the MITF pathway by exerting its effects on transcription of MITF. *(Adapted with permission from Ibrahim N, Haluska FG. Molecular pathogenesis of cutaneous melanocytic neoplasms.* Annu Rev Pathol Mech Dis. *2009;4:551-579.)*

The development of multiple pathways of resistance to BRAF inhibition has also been demonstrated from cloning melanoma BRAF mutated resistant cell lines, suggesting that overcoming clinical resistance will be a formidable barrier going forward.[45]

Another mechanism of resistance is signaling through an alternative pathway, PI3K/AKT.[46] PI3K/AKT can be activated through persistence of tyrosine kinase activity of platelet-derived growth factor receptor or insulin growth factor 1 receptor, RAS signaling, RAS independent signaling, or

loss of PTEN activity, as well as other mechanisms. PI3K/AKT and MAPK pathways are co-activated in many melanomas.[47] Resistance to mutant BRAF(V600) inhibitors develops through co-option of the PI3K/AKT pathway and RAF isoform switching.[48] These mechanisms provide the foundation for combining BRAF blockade with inhibition downstream in the MAPK pathway at MEK and inhibition of the PI3K/AKT pathway at AKT or mTOR. Another interconnection between the MAPK pathway and mTOR pathway is through phosphor-ERK (pERK) activation of RSK and TORC1, and inhibition of AMPK. AMP-activated protein kinase is activated during metabolic stress and inhibits protein and fatty acid synthesis.

The AMPK-TORC pathway can be explored using metformin, a drug that activates AMPK and is used to treat type 2 diabetes. Metformin inhibits the growth of NRAS-mutant melanoma but not BRAF-mutant melanoma cells in culture.[49] BRAF-mutant melanoma drives activation of RSK and TORC1, thus overcoming the effects of metformin on AMPK and inhibition of TORC1. Metformin, through activation of AMPK, increases degradation of dual-specificity protein phosphatase (DUSP6), thereby increasing pERK, then VEGF-A, and stimulating BRAF-mutant melanoma growth in a mouse xenograft model. Blockade of VEGF-A with an antibody (bevacizumab) and combination with metformin inhibits tumor growth. These laboratory observations may have significant implications in the choice of agents to control type 2 diabetes in melanoma patients on BRAF targeted therapy and have further implications for multiagent targeted therapy.

Notch protein, so named because it controls notch formation in the wings of fruit flies, has also been implicated in melanoma formation.[50] Notch signaling has been shown to suppress both MAPK and PI3K/AKT pathways indirectly.[51] Inhibition of one of these two pathways reverses the effects of Notch on melanoma progression.[52] A better understanding of the interaction between these pathways may also provide novel strategies for therapy.

Translocation of pERK to the nucleus leads to cell proliferation through CyclinD1. P16 or cyclin-dependent kinase inhibitor 2a (CDKN2A), located at chromosome 9p21, has been associated with familial melanoma syndromes.[53] Alterations in p16 have been seen in benign and dysplastic nevi, suggesting a role in early melanoma development and progression.[54] Thus, independent activation of proliferative pathways in melanoma may also occur significantly downstream of MAPK and theoretically lead to autonomous growth resistant to BRAF targeted therapy. Activating mutations in cyclin-dependent kinase 4 (CDK4) have also been shown to predispose to melanoma and represent yet another checkpoint that may be involved with developing resistance to BRAF targeted therapy.[55]

Melanocortin receptors are G-protein–coupled receptors and comprise a family of five different receptors. When melanocyte-stimulating hormone (α-MSH) binds to MC1R, cyclic adenosine monophosphate is generated and leads to transcription of the microphthalmia transcription factor (MITF), leading to pigment production. Low levels of MITF in melanoma cells signal proliferation and survival.[56] MC1R polymorphisms have been associated with increased risk for melanoma and nonmelanoma skin cancer. Phospho-ERK phosphorylates MITF, and pMITF is degraded through the ubiquitin pathway. Constitutive activation of pERK through mutated BRAF subsequently leads to low expression of MITF and enhances melanoma cell proliferation and survival, most likely through interaction with CDKN2a and BCL-2.[57] This pathway as a role in development of BRAF targeted therapy is not yet well elucidated, but has at least theoretical implications. MITF is also controlled by c-Kit, which is discussed later.

Heat shock protein 90 (HSP90) cooperates with its co-chaperone Cdc37 in supporting a variety of protein kinases involved with cancer progression, including BRAF. HSP90 inhibitors have had little single-agent activity in clinical trials. HSP activity across the spectrum of resistant pathways involved with BRAF inhibitors suggests a role for the combination of these agents. In cell culture experiments as well as in mouse xenograft models, HSP90 inhibitor XL888 was demonstrated to reverse vemurafenib resistance and was associated with degradation of other secondary receptor tyrosine kinases and their downstream constituents.[58]

c-Kit

c-Kit mutations are rare in melanoma and are seen most commonly in tumors derived from mucosal and acral areas or in melanomas associated with sun-damaged skin. c-Kit is involved with the melanocyte pigmentary pathway through activation of MITF. As noted previously, imatinib is a protein tyrosine kinase inhibitor that blocks protein phosphorylation by the fusion protein BCR-abl in chronic myelogenous leukemia and blocks downstream c-KIT signaling in gastrointestinal stromal tumors. Thus, there is a rationale for using imatinib in the subset of patients whose melanoma overexpresses or has mutations in c-Kit. In a Phase II study of imatinib in 43 patients with metastatic melanoma and aberrations in c-KIT, 23% had objective partial responses, and the median progression-free survival was 3.5 months.[23] Of interest is the association of benefit with mutations in exon 11 or exon 13 of c-Kit, with 9 of the 10 responding patients having these mutations. Sunitinib, another agent that inhibits mutant c-Kit, has shown similar results, with response seen in melanomas expressing mutant c-Kit and much less

so in tumors with overexpression of c-Kit.[59] Resistance to c-Kit targeted therapy has been associated with the development of NRAS mutations.[59]

Epigenetic Pathways: MicroRNA

MicroRNAs (miRNAs) are single-stranded noncoding nucleotide sequences about 22 bases long. MiRNAs are generated through a double-stranded precursor that undergoes cleavage by Drosha (RNase type III endonuclease) to an approximately 70-nucleotide unit.[60] Drosha and the associated dsRNA binding protein DGCR8 complex are transported to the nucleus and cleaved by Dicer (RNase type III endonuclease) to the approximately 22-nucleotide double-stranded miRNA. One strand of the miRNA binds to the 3′-untranslated region of messenger (m) RNA, thereby blocking translation and causing cleavage and destruction of the mRNA.[61,62]

MiRNAs are involved with regulation of a number of melanoma-related growth and proliferation pathways and are thus potential targets for therapy. Although the gain or loss of many miRNAs is shared across tumor types, a number of miRNAs are more specifically associated with melanoma.[63,64] MiR-137, located on chromosome region 1p22, and miR-182 (7q31-34) are putative negative regulators of MITF.[65,66] MITF, in turn, regulates the transcription of miR-221 and miR-222.[67] MiR-221/222 are located on chromosome X and function to inhibit expression of c-Kit receptor. Suppression of miR-221/222 with anti-mRNAs in melanoma cell lines resulted in decreasing melanoma cell proliferation and migration.[67]

MiRNAs can be obtained from archival tissues such as blood and serum, allowing them to be considered as biomarkers and potential targets for new therapeutics.[68,69] For example, high expression of miR-15b was found to correlate with poor survival in melanoma patients.[70] The soybean isoflavone genistein inhibits human uveal melanoma cell growth in culture and in a murine model and is associated with alteration of miR-27a, again suggesting that targeting of miRs may have therapeutic importance.[71] MiR-193b is downregulated in human melanoma cell lines.[72] When miR-193b was transfected back into these cells, proliferation was suppressed because of miR-193b directly downregulating cyclin D1.

Paratumoral Pathways

The tumor microenvironment is composed of vascular, stromal, and immune cells that have an intimate spatial relationship with the cancer cell and influence cancer development and progression. As we gain more understanding of these interactions, these paratumoral pathways become targets for therapy.

It is well recognized that fibroblasts are associated with many tumors and that these cells can play a contributing role in cancer progression. Tumor-infiltrating fibroblasts (TIF) are typically spindle-like and express α-smooth muscle actin, resembling myofibroblasts. These cells can express transforming growth factor-β (TGFβ), VEGF, and provide extracellular matrix, all of which support tumor growth. In a 3D coculture model, fibroblasts migrate to and infiltrate human melanoma spheroids within 7 days through melanoma-derived motility factors.[73] These fibroblasts are active and produce extracellular matrix. Following targeted therapy, for example, with an EGFR inhibitor for lung cancer, a flare phenomenon has been observed with rapid progression of cancer. Similar observations have also been noted in vemurafenib-treated melanoma patients. TIFs have been proposed as one component contributing to this phenomenon. The role of these TIFs in producing a niche for melanoma stem survival is unexplored to date, but may provide a mechanism for establishment of a resistant phenotype.

Neoangiogenesis is another paratumoral event required for the establishment and progression of cancer. The pioneering work in this area was led by the late Judah Folkman. Following anecdotal reports of alpha interferon causing regression of benign hemangiomas in infants, a study of 20 subjects demonstrated the significant activity of interferon as an anti-angiogenesis therapy.[74] The role of anti-angiogenesis therapy is now well established in a number of tumor types, although its role in melanoma is not yet definitively demonstrated. The BEAM trial in melanoma evaluated the role of adding bevacizumab (blocking anti-VEGF antibody) to carboplatin and paclitaxel in a randomized Phase II study.[75] Outcomes for the 214 treated patients demonstrate a trend toward improvement in response and median progression-free survival (PFS) (16.4% response and 4.2 months PFS for chemotherapy vs. 25.5% response and 5.6 months for chemotherapy + bevacizumab). A trial of sorafenib with either temsirolimus (an mTOR inhibitor) or tipifarnib (a farnesyl transferase inhibitor required for RAS activation) also failed to show a difference between the arms or improved response or PFS compared to historical controls.[76] Although angiogenesis is an important component of melanoma and VEGF has been implicated, studies to date have failed to show that modification of this pathway results in significant clinical benefit.

Regulation of the immune system is another area of great expectation in melanoma. IFN alfa-2b and peg-IFN alfa-2b are the only agents thus far that have shown clinical benefit in the surgical adjuvant setting of melanoma

patients with high risk for recurrence.[20,77] High-dose IL-2 has an established role in patients with good performance status who have metastatic melanoma, with a small percentage of patients (approximately 5%) reaching complete and durable remissions.[21,78] The understanding of immune regulatory checkpoints has provided a deeper understanding of immune response in cancer and therapeutic targets. Cytotoxic T lymphocyte antigen 4 (CTLA-4), a member of the immunoglobulin superfamily, has been identified as a co-stimulatory molecule involved with negative control.[79,80] Anti-CTLA-4 blocking antibodies have been developed that led to clinical trials assessing their role in the treatment of melanoma.[81,82]

Preliminary data suggested benefit of CTLA-4 blockade, and subsequent randomized trials confirmed the activity of ipilimumab in the treatment of patients with metastatic melanoma.[24,83,84] The first study randomized patients between ipilimumab at 3 mg/kg combined with gp100 vaccine, ipilimumab alone, or gp100 vaccine alone.[24,] Six hundred seventy-six previously treated patients with metastatic melanoma who expressed HLA-A*201 were assigned to ipilimumab and gp100 peptide vaccine, ipilimumab alone or gp100 vaccine alone in a 3:1:1 ratio.[24] Ipilimumab was given at 3 mg/kg every 3 weeks for up to four cycles. The ipilimumab arms had a median survival of 10 to 10.1 months compared to 6.4 months for the arm given gp100 vaccine alone. There was a 2.1% study-related mortality rate. Three patients (0.6%) on the ipilimumab arms entered a complete remission (CR). The second randomized study evaluated ipilimumab 10 mg/kg every 3 weeks with dacarbazine versus dacarbazine alone with placebo.[84] Ipilimumab or placebo was continued every 12 weeks for stable or responding patients. Five hundred two previously untreated patients were randomized in a 1:1 ratio. Median overall survival showed an improvement in the ipilimumab-dacarbazine arm from 9.1 to 11.2 months. Survival was improved in the combination arm at 1 year (47.3% vs. 36.3%), 2 years (28.5% vs. 17.9%), and 3 years (20.8% vs. 12.2%). Four patients (0.4%) had a CR in the combination arm compared to two in the dacarbazine-placebo arm, and no treatment-related deaths were reported.

It has been generally believed that immune therapy is not effective for the treatment of brain metastasis. Nevertheless, 72 patients with melanoma brain metastases were treated with ipilimumab at 10 mg/kg every 3 weeks for 24 weeks with responding patients eligible for maintenance in a Phase II study.[85] In asymptomatic patients, 12 of 51 patients obtained control of their brain metastases and 8 had objective responses. In this cohort of patients, the median overall survival was 7 months with 26% of patients alive at 24 months. This study provides the first evidence of immune therapy benefit for melanoma brain metastasis.

Engagement by antigen of the T-cell receptor (TCR)-CD3 complex provides the first signal to the T cell but is not sufficient for activation. The necessary second signal is provided by CD28 binding to its ligand B7 (1 and 2 or CD80 and CD86, respectively) on antigen-presenting cells such as dendritic cells.[86] The formation of TCR-CD3 complex and CD28 in combination with co-receptors CD4 or CD8 forms the immunological synapse.[87] Once engaged, the co-receptors that are associated with the protein tyrosine kinase LCK, phosphorylates the immune receptor tyrosine-based activation motifs (ITAMS) in the TCRζ chain initiating a cascade. This cascade includes ζ-chain–associated protein kinase (ZAP), SYK (spleen tyrosine kinase), LAT (linker for activation of T cells), and SLP76 (SRC homology 2 (CH2)-domain-containing leukocyte protein of 76 Da), which phosphorylates phospholipase Cγ1, activating the TEC family of kinases. This increases intracellular Ca^{2+}, which leads to activation of transcription factors. CTLA-4 is expressed on activated or memory T cells and has a 50- to 100-fold higher binding avidity to B7 than CD28, most likely because CTLA-4 dimer binds two bivalent B7 molecules, whereas CD28 binds to a single B7 domain.[88,89] Engagement of CTLA-4 leads to termination of the T-cell response. A number of different models have been proposed to explain the mechanism by which CTLA-4 controls regulation. Both CD28 and CTLA-4 bind PI3K at a motif that closely resembles a similar motif on growth factor receptors. CTLA-4 does not have binding domains for LCK or growth-factor receptor 2 (GRB2) whereas CD28 does. This may be one explanation of CTLA-4 activation.

It has long been the practice for oncologists to combine multiple approaches to treat patients with cancer. Thus, the use of cytoreductive surgery followed by radiation and drug therapy has become commonplace. Combining drug therapy using non–cross-resistant multiagent combinations with different mechanisms of action has also become a standard approach for many cancers. This concept is supported by mathematical modeling and observations of clinical benefit.[90-93] There is good rationale for combining targeted and immune therapies in patients with melanoma, based on the recent success. The magnitude and speed of response seen with targeted therapy may affect immune pathways, including reducing tumor-associated immune suppression and enhancing antigen availability to dendritic cells for processing and activation.[94]

The expanding list of agents available for regulating immune and signaling pathways provides tools for exploring this complex cross talk. A mutant BRAF melanoma cell line treated with a BRAF target blocker upregulates melanoma differentiation antigens and can be better recognized and killed by melanoma-specific cytotoxic T cells.[95] Although MEK inhibition also upregulates melanoma differentiation antigens in this model, blocking MEK also inhibits T-cell killing whereas BRAF blockade does not. An increase in

tumor-infiltrating T cells (TILs) has been observed in tumors from patients treated with vemurafenib.[96] We have observed a similar TIL infiltration in a mouse model of BRAF mutant melanoma treated with BRAF blockade (Turk MJ, personal communication). Targeted agents can have both a negative and positive interaction with immune regulation.[97] In addition to the BRAF(V600E) targeted agents, other melanoma-relevant agents that can be considered for combination with immune therapy include imatinib, which blocks c-kit and can also decrease indoleamine dioxygenase (IDO), a negative regulator of immune function, as well as promote dendritic and natural killer cell communication; bevacizumab, a VEGF-neutralizing antibody, which increases DC maturation; bortezomib, a proteasome inhibitor, which can sensitize tumor to immune-mediated killing; PI3K-AKT inhibitors that also enhance tumor cell sensitivity to immune-mediated killing; and HSP90 inhibitors, which can decrease immune suppression and increase NK cell targets on the tumor.[97] As discussed earlier, understanding the molecular pathways of immune cells in the context of cancer will also lead to new strategies of targeted therapy directed at enhancing immune activation and inhibiting regulation.[98,99]

Conclusions

Recent updates of ongoing clinical trials provided with BRAF inhibitors and ipilimumab demonstrate the promise of these new therapeutic tools as well as their limitations. High response rates to vemurafenib and dabrafenib are met with the development of early clinical resistance.[100,101] Although both progression-free survival and overall survival are now extended in patients with metastatic melanoma with BRAF(V600E) mutation, the median overall survival in the BRIM3 trial of 13.2 months is short and demonstrates the need for strategies to convert the initial response to long-term survivorship in a higher percentage of patients.[101,102] The challenge may be met with the identification of resistant pathways and the development of additional agents targeted to these pathways, such as the combination of MEK and BRAF inhibitors. The pipeline in the pharmaceutical industry is full of agents that target MEK, ERK, and others. MiRNA inhibitors are also being developed. Our knowledge of how these signals intersect with the molecular pathway and how miRNAs are regulated should provide additional therapeutic strategies that will need to be tested in the clinical setting.

Another approach is to use pathway inhibitors early in the setting of high-risk stage II and III disease. The questions of which pathway, which agent, and for what duration of therapy can only be answered through the experience gained in clinical trials. The early development of heterogeneity of molecular profiles within cancer[103] may ultimately allow for aggressive and resistant clones to threaten patients' lives and prove to be a barrier that will require combination of therapeutic strategies.

The low objective response rate noted with anti-CTLA4 antibody is counterbalanced by the observation of long complete remissions seen in a small percentage of patients no longer needing additional therapy. This improvement of the tail of the curve is an important concept and should not be underestimated as we seek to enhance the long-term goal of cure of metastatic melanoma.[104] Our understanding of the complex inflammatory and regulatory networks of immune pathways has led to the development of new therapeutic tools that are being and will need to be explored through clinical trials with correlative biological studies. Our recognition of the molecular pathways activated in immune cells from patients with cancer[98,99] will provide new strategies to regulate those pathways.

There is a long history of recognizing that the inflammatory responses in cancers treated with chemotherapy contribute to benefit. We have now identified inflammatory responses in tumors treated with the new targeted therapies, providing an additional rationale for combining these therapeutic strategies.

We have begun to unravel the molecular mysteries of melanoma carcinogenesis, progression, and metastasis, as well as the molecular pathways regulating the host response to this disease. Through judicious and thoughtful manipulation of these pathways, melanoma will likely be tamed to a chronic illness that does not cause death or can be cured outright.

Key References

7. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med*. 1996;2:561-566.
8. Jemal A, Saraiya M, Patel P, et al. Recent trends in cutaneous melanoma incidence and death rates in the United States, 1992-2006. *J Am Acad Dermatol*. 2011;65: S17-S25, e1-3.
9. Merrill RM. Risk-adjusted melanoma skin cancer incidence rates in Whites (United States). *Melanoma Res*. 2011;21:535-540.
10. Soong SJ, Ding S, Coit D, et al. Predicting survival outcome of localized melanoma: an electronic prediction tool based on the AJCC Melanoma Database. *Ann Surg Oncol*. 2010;17:2006-2014.
11. Balch CM, Gershenwald JE, Soong SJ, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;27:6199-6206.
12. Tadokoro T, Yamaguchi Y, Batzer J, et al. Mechanism of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *J Invest Dermatol*. 2005;124:1326-1332.

16. Duray PH, Ernstoff MS. Dysplastic nevus in histologic contiguity with acquired nonfamilial melanoma. Clinicopathologic experience in a 100-bed hospital. *Arch Dermatol*. 1987;123:80-84.
17. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med*. 2005;353:2135-2147.
18. Rigel DS, Friedman RJ, Kopf AW, et al. ABCDE—an evolving concept in the early detection of melanoma. *Arch Dermatol*. 2005;141:1032-1034.
20. Kirkwood JM, Strawderman MH, Ernstoff MS, et al. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol*. 1996;14:7-17.
22. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364:2507-2516.
23. Guo J, Si L, Kong Y, et al. Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-Kit mutation or amplification. *J Clin Oncol*. 2011;29:2904-2909.
24. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
26. Saldanha G, Potter L, Daforno P, et al. Cutaneous melanoma subtypes show different BRAF and NRAS mutation frequencies. *Clin Cancer Res*. 2006;12:4499-4505.
29. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19-20.
30. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 2004;64:7099-7109.
31. Eisen T, Ahmad T, Flaherty KT, et al. Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br J Cancer*. 2006;95:581-586.
32. Amaravadi RK, Schuchter LM, McDermott DF, et al. Phase II trial of temozolomide and sorafenib in advanced melanoma patients with or without brain metastases. *Clin Cancer Res*. 2009;15:7711-7718.
33. Eisen T, Marais R, Affolter A, et al. Sorafenib and dacarbazine as first-line therapy for advanced melanoma: phase I and open-label phase II studies. *Br J Cancer*. 2011;105:353-359.
34. Tsai J, Lee JT, Wang W, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A*. 2008;105:3041-3046.
35. Liu Y, Gray NS. Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol*. 2006;2:358-364.
36. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
37. Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med*. 2012;366:207-215.
38. Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med*. 2012;366:707-714.
39. Hong DS, Vence L, Falchook G, et al. BRAF(V600) Inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency. *Clin Cancer Res*. 2012;18:2326-2335.
40. Poulikakos PI, Persaud Y, Janakiraman M, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature*. 2011;480:387-390.
41. Montagut C, Sharma SV, Shioda T, et al. Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res*. 2008;68:4853-4861.
42. Johannessen CM, Boehm JS, Kim SY, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature*. 2010;468:968-972.
43. Nazarian R, Shi H, Wang Q, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature*. 2010;468:973-977.
44. Wagle N, Emery C, Berger MF, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol*. 2011;29:3085-3096.
45. Gowrishankar K, Snoyman S, Pupo GM, et al. Acquired resistance to BRAF inhibition can confer cross-resistance to combined BRAF/MEK inhibition. *J Invest Dermatol*. 2012;132:1850-1859.
46. Shimizu T, Tolcher AW, Papadopoulos KP, et al. The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res*. 2012;18:2316-2325.
47. Russo AE, Torrisi E, Bevelacqua Y, et al. Melanoma: molecular pathogenesis and emerging target therapies (review). *Int J Oncol*. 2009;34:1481-1489.
48. Villanueva J, Vultur A, Herlyn M. Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options. *Cancer Res*. 2011;71:7137-7140.
49. Martin MJ, Hayward R, Viros A, et al. Metformin accelerates the growth of BRAFV600E-driven melanoma by upregulating VEGF-A. *Cancer Discov*. 2012;2:344-355.
50. Hoek K, Rimm DL, Williams KR, et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res*. 2004;64:5270-5282.
51. Liu ZJ, Xiao M, Balint K, et al. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res*. 2006;66:4182-4190.
52. Liu ZJ, Xiao M, Balint K, et al. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *FASEB J*. 2006;20:1009-1011.
54. Tran TP, Titus-Ernstoff L, Perry AE, et al. Alteration of chromosome 9p21 and/or p16 in benign and dysplastic nevi suggests a role in early melanoma progression (United States). *Cancer Causes Control*. 2002;13:675-682.
56. Wellbrock C, Marais R. Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. *J Cell Biol*. 2005;170:703-708.
57. Wellbrock C, Rana S, Paterson H, et al. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. *PLoS One*. 2008;3:e2734.
58. Paraiso KH, Haarberg HE, Wood E, et al. The HSP90 inhibitor XL888 overcomes BRAF inhibitor resistance mediated through diverse mechanisms. *Clin Cancer Res*. 2012;18:2502-2514.
59. Minor DR, Kashani-Sabet M, Garrido M, et al. Sunitinib therapy for melanoma patients with KIT mutations. *Clin Cancer Res*. 2012;18:1457-1463.
60. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003;425:415-419.
63. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res*. 2007;67:2456-2468.
64. Zhang L, Huang J, Yang N, et al. MicroRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci U S A*. 2006;103:9136-9141.
65. Segura MF, Hanniford D, Menendez S, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A*. 2009;106:1814-1819.
66. Bemis LT, Chen R, Amato CM, et al. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. *Cancer Res*. 2008;68:1362-1368.
67. Felicetti F, Errico MC, Segnalini P, et al. MicroRNA-221 and -222 pathway controls melanoma progression. *Expert Rev Anticancer Ther*. 2008;8:1759-1765.
68. Cortez MA, Calin GA. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin Biol Ther*. 2009;9:703-711.
69. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA*. 2007;13:1668-1674.
70. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. *Int J Cancer*. 2010;126:2553-2562.

71. Sun Q, Cong R, Yan H, et al. Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression. *Oncol Rep*. 2009;22:563-567.
72. Chen J, Feilotter HE, Pare GC, et al. MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. *Am J Pathol*. 2010;176:2520-2529.
73. Flach EH, Rebecca VW, Herlyn M, et al. Fibroblasts contribute to melanoma tumor growth and drug resistance. *Mol Pharm*. 2011;8:2039-2049.
75. Kim KB, Sosman JA, Fruehauf JP, et al. BEAM: a randomized phase II study evaluating the activity of bevacizumab in combination with carboplatin plus paclitaxel in patients with previously untreated advanced melanoma. *J Clin Oncol*. 2012;30:34-41.
76. Margolin KA, Moon J, Flaherty LE, et al. Randomized phase II trial of sorafenib with temsirolimus or tipifarnib in untreated metastatic melanoma (S0438). *Clin Cancer Res*. 2012;18:1129-1137.
77. Bottomley A, Coens C, Suciu S, et al. Adjuvant therapy with pegylated interferon alfa-2b versus observation in resected stage III melanoma: a phase III randomized controlled trial of health-related quality of life and symptoms by the European Organisation for Research and Treatment of Cancer Melanoma Group. *J Clin Oncol*. 2009;27:2916-2923.
78. Gunturu KS, Meehan KR, Mackenzie TA, et al. Cytokine working group study of lymphodepleting chemotherapy, interleukin-2, and granulocyte-macrophage colony-stimulating factor in patients with metastatic melanoma: clinical outcomes and peripheral-blood cell recovery. *J Clin Oncol*. 2010;28:1196-1202.
80. Allison JP, Krummel MF. The yin and yang of T cell costimulation. *Science*. 1995;270:932-933.
81. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003;100:8372-8377.
82. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol*. 2005;23:8968-8977.
83. Ernstoff MS. Self-recognition and tumor response to immunotherapy. *J Clin Oncol*. 2005;23:5875-5877.
84. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011;364:2517-2526.
85. Margolin K, Ernstoff MS, Hamid O, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol*. 2012;13:459-465.
86. Kalland ME, Oberprieler NG, Vang T, et al. T cell-signaling network analysis reveals distinct differences between CD28 and CD2 costimulation responses in various subsets and in the MAPK pathway between resting and activated regulatory T cells. *J Immunol*. 2011;187:5233-5245.
87. Smeets RL, Fleuren WW, He X, et al. Molecular pathway profiling of T lymphocyte signal transduction pathways; Th1 and Th2 genomic fingerprints are defined by TCR and CD28-mediated signaling. *BMC Immunol*. 2012;13:12.
89. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol*. 2003;3:544-556.
93. Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet*. 2007;369:29-36.
94. Schwaab T, Schwarzer A, Wolf B, et al. Clinical and immunologic effects of intranodal autologous tumor lysate-dendritic cell vaccine with aldesleukin (interleukin 2) and IFN-α2a therapy in metastatic renal cell carcinoma patients. *Clin Cancer Res*. 2009;15:4986-4992.
95. Boni A, Cogdill AP, Dang P, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer Res*. 2010;70:5213-5219.
96. Wilmott JS, Long GV, Howle JR, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. *Clin Cancer Res*. 2012;18:1386-1394.
97. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*. 2012;12:237-251.
98. Schwarzer A, Fisher JL, Schwaab T, et al. Regulatory T-cells and associated pathways in metastatic renal cell carcinoma (mRCC) patients undergoing DC-vaccination and cytokine-therapy. *PLoS One*. 2012;7:e46600.
99. Wolf B, Schwarzer A, Cote AL, et al. Gene expression profile of peripheral blood lymphocytes from metastatic renal cell cancer patients treated with IL-2, interferon-alfa and dendritic cell vaccine. *PLoS One*. 2012;7:e50221.
100. Hauschild A, Grob JJ, Demidov LV, et al. Phase III, randomized, open-label, multicenter trial (BREAK-3) comparing the BRAF kinase inhibitor dabrafenib (GSK2118436) with dacarbazine (DTIC) in patients with BRAFV600E-mutated melanoma. *J Clin Oncol*. 2012;30: suppl;abstr lba8500.
101. Chapman PB, Hauschild A, Robert C, et al. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial comparing BRAF inhibitor vemurafenib (vem) with dacarbazine (DTIC) in previously untreated patients with BRAFV600E-mutated melanoma. *J Clin Oncol*. 2012;30: suppl;abstr 8502.
102. Ernstoff MS. Been there, not done that—melanoma in the age of molecular therapy. *N Engl J Med*. 2011;364:2547-2548.
103. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
104. Gould SJ. The median isn't the message. *Discover*. 1985;6:40-42.

References

1. Rudkin CT, Hungerford DA, Nowell PC. DNA contents of chromosome Ph1 and chromosome 21 in human chronic granulocytic leukemia. *Science*. 1964;144:1229-1231.
2. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243:290-293.
3. Rosenberg N, Baltimore D. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. *J Exp Med*. 1976;143:1453-1463.
4. Witte ON, Rosenberg N, Paskind M, et al. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblast and lymphoid cells. *Proc Natl Acad Sci U S A*. 1978;75:2488-2492.
5. de Klein A, van Kessel AG, Grosveld G, et al. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature*. 1982;300:765-767.
6. Daley GQ, McLaughlin J, Witte ON, et al. The CML-specific P210 bcr/abl protein, unlike v-abl, does not transform NIH/3T3 fibroblasts. *Science*. 1987;237:532-535.
7. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med*. 1996;2:561-566.
8. Jemal A, Saraiya M, Patel P, et al. Recent trends in cutaneous melanoma incidence and death rates in the United States, 1992-2006. *J Am Acad Dermatol*. 2011;65: S17-S25, e1-3.
9. Merrill RM. Risk-adjusted melanoma skin cancer incidence rates in Whites (United States). *Melanoma Res*. 2011;21:535-540.
10. Soong SJ, Ding S, Coit D, et al. Predicting survival outcome of localized melanoma: an electronic prediction tool based on the AJCC Melanoma Database. *Ann Surg Oncol*. 2010;17:2006-2014.
11. Balch CM, Gershenwald JE, Soong SJ, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;27:6199-6206.
12. Tadokoro T, Yamaguchi Y, Batzer J, et al. Mechanism of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *J Invest Dermatol*. 2005;124:1326-1332.
13. Kirkham N, Price ML, Gibson B, et al. Type VII collagen antibody LH 7.2 identifies basement membrane characteristics of thin malignant melanomas. *J Pathol*. 1989;157:243-247.
14. Duray PH, DerSimonian R, Barnhill R, et al. An analysis of interobserver recognition of the histopathologic features of dysplastic nevi from a mixed group of nevomelanocytic lesions. *J Am Acad Dermatol*. 1992;27:741-749.
15. Titus-Ernstoff L, Barnhill RL, Duray PH, et al. Dysplastic nevi in relation to superficial spreading melanoma. *Cancer Epidemiol Biomarkers Prev*. 1993;2:99-101.
16. Duray PH, Ernstoff MS. Dysplastic nevus in histologic contiguity with acquired nonfamilial melanoma. Clinicopathologic experience in a 100-bed hospital. *Arch Dermatol*. 1987;123:80-84.
17. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med*. 2005;353:2135-2147.
18. Rigel DS, Friedman RJ, Kopf AW, et al. ABCDE—an evolving concept in the early detection of melanoma. *Arch Dermatol*. 2005;141:1032-1034.
19. Chapman PB, Einhorn LH, Meyers ML, et al. Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *J Clin Oncol*. 1999;17:2745-2751.
20. Kirkwood JM, Strawderman MH, Ernstoff MS, et al. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *J Clin Oncol*. 1996;14:7-17.
21. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol*. 1999;17:2105-2116.
22. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364:2507-2516.
23. Guo J, Si L, Kong Y, et al. Phase II, open-label, single-arm trial of imatinib mesylate in patients with metastatic melanoma harboring c-Kit mutation or amplification. *J Clin Oncol*. 2011;29:2904-2909.
24. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
25. Demunter A, Stas M, Degreef H, et al. Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. *J Invest Dermatol*. 2001;117:1483-1489.
26. Saldanha G, Potter L, Daforno P, et al. Cutaneous melanoma subtypes show different BRAF and NRAS mutation frequencies. *Clin Cancer Res*. 2006;12:4499-4505.
27. Mason CS, Springer CJ, Cooper RG, et al. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J*. 1999;18:2137-2148.
28. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.
29. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19-20.
30. Wilhelm SM, Carter C, Tang L, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 2004;64:7099-7109.
31. Eisen T, Ahmad T, Flaherty KT, et al. Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br J Cancer*. 2006;95:581-586.
32. Amaravadi RK, Schuchter LM, McDermott DF, et al. Phase II trial of temozolomide and sorafenib in advanced melanoma patients with or without brain metastases. *Clin Cancer Res*. 2009;15:7711-7718.
33. Eisen T, Marais R, Affolter A, et al. Sorafenib and dacarbazine as first-line therapy for advanced melanoma: phase I and open-label phase II studies. *Br J Cancer*. 2011;105:353-359.
34. Tsai J, Lee JT, Wang W, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A*. 2008;105:3041-3046.
35. Liu Y, Gray NS. Rational design of inhibitors that bind to inactive kinase conformations. *Nat Chem Biol*. 2006;2:358-364.
36. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
37. Su F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *N Engl J Med*. 2012;366:207-215.
38. Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med*. 2012;366:707-714.
39. Hong DS, Vence L, Falchook G, et al. BRAF(V600) Inhibitor GSK2118436 targeted inhibition of mutant BRAF in cancer patients does not impair overall immune competency. *Clin Cancer Res*. 2012;18:2326-2335.
40. Poulikakos PI, Persaud Y, Janakiraman M, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature*. 2011;480:387-390.
41. Montagut C, Sharma SV, Shioda T, et al. Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res*. 2008;68:4853-4861.
42. Johannessen CM, Boehm JS, Kim SY, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature*. 2010;468:968-972.
43. Nazarian R, Shi H, Wang Q, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature*. 2010;468:973-977.
44. Wagle N, Emery C, Berger MF, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol*. 2011;29:3085-3096.
45. Gowrishankar K, Snoyman S, Pupo GM, et al. Acquired resistance to BRAF inhibition can confer cross-resistance to combined BRAF/MEK inhibition. *J Invest Dermatol*. 2012;132:1850-1859.
46. Shimizu T, Tolcher AW, Papadopoulos KP, et al. The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res*. 2012;18:2316-2325.

47. Russo AE, Torrisi E, Bevelacqua Y, et al. Melanoma: molecular pathogenesis and emerging target therapies (review). *Int J Oncol*. 2009;34:1481-1489.
48. Villanueva J, Vultur A, Herlyn M. Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options. *Cancer Res*. 2011;71:7137-7140.
49. Martin MJ, Hayward R, Viros A, et al. Metformin accelerates the growth of BRAFV600E-driven melanoma by upregulating VEGF-A. *Cancer Discov*. 2012;2:344-355.
50. Hoek K, Rimm DL, Williams KR, et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res*. 2004;64:5270-5282.
51. Liu ZJ, Xiao M, Balint K, et al. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res*. 2006;66:4182-4190.
52. Liu ZJ, Xiao M, Balint K, et al. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *FASEB J*. 2006;20:1009-1011.
53. Fountain JW, Karayiorgou M, Ernstoff MS, et al. Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc Natl Acad Sci U S A*. 1992;89:10557-10561.
54. Tran TP, Titus-Ernstoff L, Perry AE, et al. Alteration of chromosome 9p21 and/or p16 in benign and dysplastic nevi suggests a role in early melanoma progression (United States). *Cancer Causes Control*. 2002;13:675-682.
55. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet*. 1996;12:97-99.
56. Wellbrock C, Marais R. Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. *J Cell Biol*. 2005;170:703-708.
57. Wellbrock C, Rana S, Paterson H, et al. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. *PLoS One*. 2008;3:e2734.
58. Paraiso KH, Haarberg HE, Wood E, et al. The HSP90 inhibitor XL888 overcomes BRAF inhibitor resistance mediated through diverse mechanisms. *Clin Cancer Res*. 2012;18:2502-2514.
59. Minor DR, Kashani-Sabet M, Garrido M, et al. Sunitinib therapy for melanoma patients with KIT mutations. *Clin Cancer Res*. 2012;18:1457-1463.
60. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003;425:415-419.
61. Bernstein E, Caudy AA, Hammond SM, et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001;409:363-366.
62. Hammond SM, Boettcher S, Caudy AA, et al. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science*. 2001;293:1146-1150.
63. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res*. 2007;67:2456-2468.
64. Zhang L, Huang J, Yang N, et al. MicroRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci U S A*. 2006;103:9136-9141.
65. Segura MF, Hanniford D, Menendez S, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A*. 2009;106:1814-1819.
66. Bemis LT, Chen R, Amato CM, et al. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. *Cancer Res*. 2008;68:1362-1368.
67. Felicetti F, Errico MC, Segnalini P, et al. MicroRNA-221 and -222 pathway controls melanoma progression. *Expert Rev Anticancer Ther*. 2008;8:1759-1765.
68. Cortez MA, Calin GA. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin Biol Ther*. 2009;9:703-711.
69. Xi Y, Nakajima G, Gavin E, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA*. 2007;13:1668-1674.
70. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. *Int J Cancer*. 2010;126:2553-2562.
71. Sun Q, Cong R, Yan H, et al. Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression. *Oncol Rep*. 2009;22:563-567.
72. Chen J, Feilotter HE, Pare GC, et al. MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. *Am J Pathol*. 2010;176:2520-2529.
73. Flach EH, Rebecca VW, Herlyn M, et al. Fibroblasts contribute to melanoma tumor growth and drug resistance. *Mol Pharm*. 2011;8:2039-2049.
74. Ezekowitz RA, Mulliken JB, Folkman J. Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med*. 1992;326:1456-1463.
75. Kim KB, Sosman JA, Fruehauf JP, et al. BEAM: a randomized phase II study evaluating the activity of bevacizumab in combination with carboplatin plus paclitaxel in patients with previously untreated advanced melanoma. *J Clin Oncol*. 2012;30:34-41.
76. Margolin KA, Moon J, Flaherty LE, et al. Randomized phase II trial of sorafenib with temsirolimus or tipifarnib in untreated metastatic melanoma (S0438). *Clin Cancer Res*. 2012;18:1129-1137.
77. Bottomley A, Coens C, Suciu S, et al. Adjuvant therapy with pegylated interferon alfa-2b versus observation in resected stage III melanoma: a phase III randomized controlled trial of health-related quality of life and symptoms by the European Organisation for Research and Treatment of Cancer Melanoma Group. *J Clin Oncol*. 2009;27:2916-2923.
78. Gunturu KS, Meehan KR, Mackenzie TA, et al. Cytokine working group study of lymphodepleting chemotherapy, interleukin-2, and granulocyte-macrophage colony-stimulating factor in patients with metastatic melanoma: clinical outcomes and peripheral-blood cell recovery. *J Clin Oncol*. 2010;28:1196-1202.
79. Brunet JF, Denizot F, Luciani MF, et al. A new member of the immunoglobulin superfamily—CTLA-4. *Nature*. 1987;328:267-270.
80. Allison JP, Krummel MF. The yin and yang of T cell costimulation. *Science*. 1995;270:932-933.
81. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003;100:8372-8377.
82. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol*. 2005;23:8968-8977.
83. Ernstoff MS. Self-recognition and tumor response to immunotherapy. *J Clin Oncol*. 2005;23:5875-5877.
84. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011;364:2517-2526.
85. Margolin K, Ernstoff MS, Hamid O, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *Lancet Oncol*. 2012;13:459-465.
86. Kalland ME, Oberprieler NG, Vang T, et al. T cell-signaling network analysis reveals distinct differences between CD28 and CD2 costimulation responses in various subsets and in the MAPK pathway between resting and activated regulatory T cells. *J Immunol*. 2011;187:5233-5245.
87. Smeets RL, Fleuren WW, He X, et al. Molecular pathway profiling of T lymphocyte signal transduction pathways; Th1 and Th2 genomic fingerprints are defined by TCR and CD28-mediated signaling. *BMC Immunol*. 2012;13:12.
88. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol*. 2001;1:220-228.
89. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol*. 2003;3:544-556.
90. Day RS. Treatment sequencing, asymmetry, and uncertainty: protocol strategies for combination chemotherapy. *Cancer Res*. 1986;46:3876-3885.
91. Goldie JH, Coldman AJ, Gudauskas GA. Rationale for the use of alternating non-cross-resistant chemotherapy. *Cancer Treat Rep*. 1982;66:439-449.
92. Santoro A, Bonadonna G, Bonfante V, et al. Alternating drug combinations in the treatment of advanced Hodgkin's disease. *N Engl J Med*. 1982;306:770-775.
93. Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet*. 2007;369:29-36.
94. Schwaab T, Schwarzer A, Wolf B, et al. Clinical and immunologic effects of intranodal autologous tumor lysate-dendritic cell vaccine with aldesleukin (interleukin 2) and IFN-α2a therapy in metastatic renal cell carcinoma patients. *Clin Cancer Res*. 2009;15:4986-4992.

95. Boni A, Cogdill AP, Dang P, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer Res*. 2010;70:5213-5219.
96. Wilmott JS, Long GV, Howle JR, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. *Clin Cancer Res*. 2012;18:1386-1394.
97. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*. 2012;12:237-251.
98. Schwarzer A, Fisher JL, Schwaab T, et al. Regulatory T-cells and associated pathways in metastatic renal cell carcinoma (mRCC) patients undergoing DC-vaccination and cytokine-therapy. *PLoS One*. 2012;7:e46600.
99. Wolf B, Schwarzer A, Cote AL, et al. Gene expression profile of peripheral blood lymphocytes from metastatic renal cell cancer patients treated with IL-2, interferon-alfa and dendritic cell vaccine. *PLoS One*. 2012;7:e50221.
100. Hauschild A, Grob JJ, Demidov LV, et al. Phase III, randomized, open-label, multicenter trial (BREAK-3) comparing the BRAF kinase inhibitor dabrafenib (GSK2118436) with dacarbazine (DTIC) in patients with BRAFV600E-mutated melanoma. *J Clin Oncol*. 2012;30: suppl;abstr lba8500.
101. Chapman PB, Hauschild A, Robert C, et al. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial comparing BRAF inhibitor vemurafenib (vem) with dacarbazine (DTIC) in previously untreated patients with BRAFV600E-mutated melanoma. *J Clin Oncol*. 2012;30: suppl;abstr 8502.
102. Ernstoff MS. Been there, not done that—melanoma in the age of molecular therapy. *N Engl J Med*. 2011;364:2547-2548.
103. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
104. Gould SJ. The median isn't the message. *Discover*. 1985;6:40-42.

Steven I. Sherman

43 Thyroid Cancer

Cancer of the thyroid is the ninth most common malignancy diagnosed worldwide in women and 18th in both genders.[1] In the United States, the incidence has been rising faster than that of any other malignancy; in 2012, overall incidence is projected to be 56,460 persons, ranked fifth among all newly diagnosed malignancies in women.[2] Although only 1780 deaths from thyroid cancer are expected in the United States in 2012, the average age-adjusted mortality increased 0.6% per year between 1998 and 2007, most notably among men, who experienced a striking 1.6% increase per year.[3]

Derived from follicular epithelial cells, differentiated thyroid carcinomas (DTC) include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC). These cells generally retain many of the differentiated functions of normal follicular cells, including the ability to respond to stimulation from thyrotropin (TSH) and to concentrate and organify iodine. More aggressive follicular cell-derived histologies include poorly differentiated (PDTC) and anaplastic carcinomas (ATC), which are generally thought to derive from progressive dedifferentiation of DTC although they may also arise de novo. Medullary thyroid carcinoma (MTC), on the other hand, is derived from neuroendocrine C cells present typically in the upper two thirds of each thyroid lobe that lack any of the differentiated functions associated with thyroid follicular epithelial cells.

Characteristic oncogenic mutations have been identified that appear to give rise to the majority of DTC and MTC, affecting tyrosine kinase receptors and downstream signaling intermediates in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (Figures 43-1 and 43-2). Their importance is underscored by the virtual absence of overlap among the three most common mutations that all yield activation of MAPK signaling: *BRAF*, *RAS*, and *RET/PTC*. Less common mutations may be involved in the dedifferentiation steps, including loss of tumor suppressors. Further abnormalities that may contribute to tumor proliferation, invasion, and dedifferentiation may include DNA epigenetic alterations, gene amplifications, and other mechanisms that contribute to select gene overexpression. With understanding of these molecular abnormalities that underlie thyroid malignancies, appropriate targeted therapies have been introduced in the past several years and have become a new standard of therapy for advanced metastatic disease. This review focuses on those molecular abnormalities that have been most strongly associated with mechanisms of disease, clinical prognosis, and therapies.

Differentiated Thyroid Carcinoma

Receptor Tyrosine Kinases (RTKs)

Physiologically, RET is a transmembrane RTK with expression typically restricted to certain neuronal and neuroendocrine cells, the collecting duct of the kidneys, and spermatogonial stem cells, but is not normally expressed in thyroid follicular cells. In cooperation with co-receptors, RET binds ligands of the glial cell-derived neurotrophic growth factor (GDNF) family, which leads to receptor dimerization and autophosphorylation of key intracellular tyrosine residues that subsequently activate downstream signaling in multiple pathways.

Somatic chromosomal rearrangements of the *RET* gene were identified in papillary thyroid carcinomas more than 20 years ago that cause constitutive expression and activation of a RET fusion protein denoted RET/PTC.[6] To date, more than a dozen *RET/PTC* translocations have been reported, although *RET/PTC1* and *RET/PTC3* account for more than 90% of the tumors associated with the mutation. In each case, the promoter and N-terminal domain of a heterologous gene fuse with a C-terminal domain of the *RET* gene containing the tyrosine kinase functions, permitting inappropriate expression of RET kinase in thyroid follicular cells under control of the heterologous promoter. The resultant fusion protein resides in the cytosol rather than the

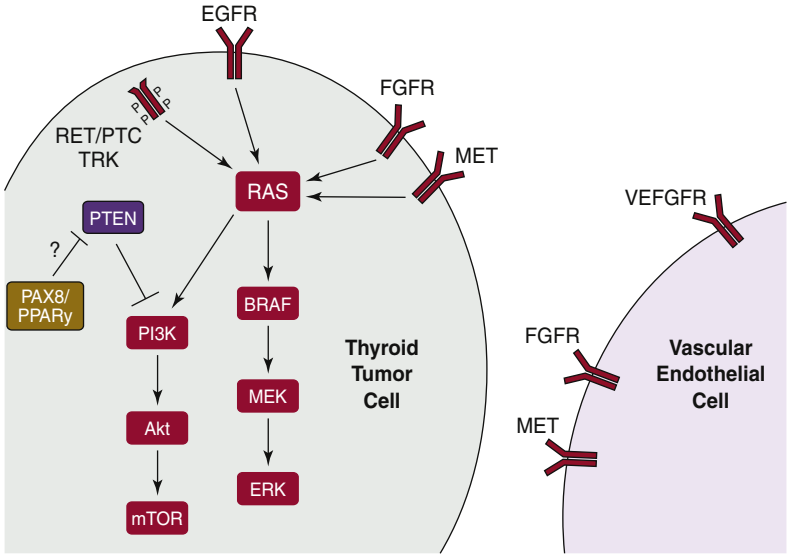


Figure 43-1 Schematic illustration of the key intracellular signaling pathways involved in the pathogenesis of differentiated thyroid carcinoma.

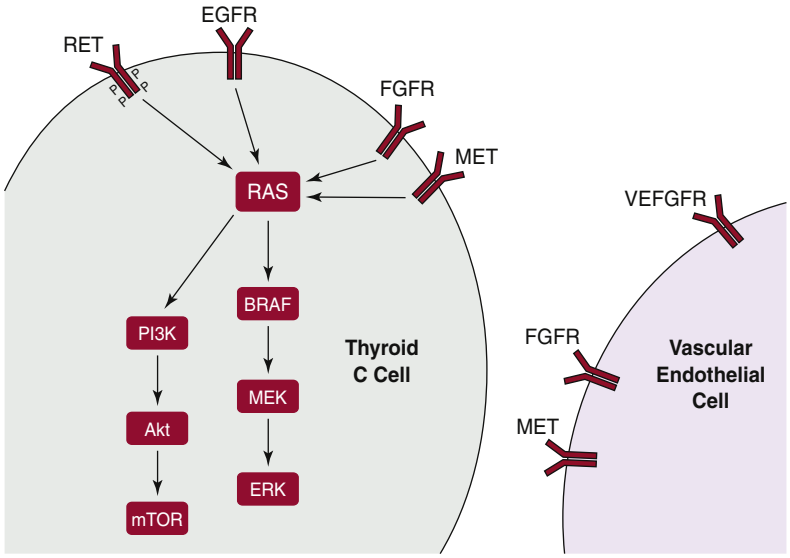


Figure 43-2 Schematic illustration of the key intracellular signaling pathways involved in the pathogenesis of medullary thyroid carcinoma.

cell membrane and is capable of ligand-independent homodimerization, thus triggering downstream signaling activation. A further mechanism to promote tumorigenesis is through cooperation between RET/PTC and another RTK, the epidermal growth factor receptor (EGFR), which is found to colocate with RET/PTC and jointly signal downstream through RAS.[7] *RET/PTC* mutations are commonly seen in about 10% to 20% of PTC (particularly the classic, solid, and almost all oxyphilic variants but less commonly in follicular and tall cell variants), and rarely in PDTC or FTC. The oncogenic role of *RET/PTC* mutations is supported by a high rate of papillary carcinomas in transgenic mice expressing either *RET/PTC1* or *RET/PTC3*, and the mutation is frequently found in occult microcarcinomas.[8]

A second group of chromosomal translocations associated with PTC comprise mutations in the *NTRK* gene that lead to formation of various *TRK* oncogenes. Similar to RET/PTC, these fusion TRK proteins combine the N terminus of one of several genes normally expressed in thyroid follicular cells with the tyrosine kinase domain of the receptor for nerve growth factor.[9] These mutations are seen in about 5% of PTC.

These chromosomal rearrangements involving tyrosine receptor genes are commonly seen following exposure to ionizing radiation and may be the leading mechanism of thyroid oncogenesis in this setting. This has been particularly true following the unfortunate experiences of the Chernobyl nuclear power plant accident as well as atomic bomb explosions in Japan.[10,11] Spatial proximity of *RET* or *NTRK* genes to the heterologous donors of the N-terminal promoter regions specific to chromosomal folding in thyroid follicular cells may permit a single radiation event to cause double-strand breaks in each gene, thus permitting the recombination event.

Other RTKs have been found to be overexpressed in DTC cells and may be relevant to disease biology. In addition to EGFR, these include platelet-derived growth factor receptor (PDGFR) α and β; vascular endothelial growth factor receptors (VEGFR) 1 and 2; fibroblast growth factor receptors (FGFR); and hepatocyte growth factor receptor (MET). Genetic abnormalities are rarely observed, and epigenetic alterations may be contributory; however, the exact mechanisms leading to overexpression are generally not known.

BRAF

Mutations of the serine-threonine kinase BRAF are the most common oncogenic abnormality reported in PTC.[13] Activated frequently in other cancers as well, BRAF has high affinity for binding and phosphorylating MEK isoforms in the MAPK pathway. Although point mutations in multiple codons have been reported to cause constitutive activation of BRAF, the most studied and most frequent is a valine-to-glutamine substitution at amino acid residue 600 (denoted V600E mutation). By destabilizing the inactive conformation of the kinase, the V600E *BRAF* mutation causes the protein to remain in a catalytically competent conformation that allows continuous phosphorylation of MEK. *BRAF* mutations are virtually never seen in benign thyroid lesions, and thus the presence of a *BRAF* mutation can be pathognomonic of a malignancy if detected in a cytologically suspicious biopsy specimen.[15] Of note, a rare translocation mutation of *BRAF* (causing fusion of the *AKAp9* gene with BRAF) has been reported to cause PTC after radiation exposure.

BRAF mutations occur in 40% to 50% of cases of PTC (especially the classical and tall-cell variants) and are also frequent in PDTC and ATC. Multiple studies describe a more aggressive phenotype associated with these mutations, including higher rates of lymph node metastases, extrathyroidal extension, poor radioiodine uptake and response to therapy, and advanced stage at presentation. Prognostically, *BRAF* mutations are associated with higher rates of recurrence and worse survival.[13,16] In a model of conditional activation of the V600E *BRAF* mutant in thyroid follicular cells, mice develop rapidly growing poorly differentiated tumors with negligible expression of thyroid-specific genes such as the sodium-iodide symporter as well as loss of iodine incorporation; these changes are reversible on inhibition of BRAF or MEK kinase functions.[17] Other downstream effects of mutant BRAF include alterations in DNA methylation and increased expression of genes associated with invasive and metastatic disease such as matrix metalloproteinases.[18]

RAS

Point mutations in *RAS* genes are among the most common oncogenic abnormalities in all cancers, and DTC is no different. Mutations in the RAS protein lead to constitutive activation through alterations in the binding affinity of the kinase for GTP or through inactivation of its intrinsic GTPase activity. Thus, mutant RAS can signal downstream through both the MAPK and PI3K/Akt pathways without upstream activation derived from ligand-bound RTK. All three *RAS* genes (*H-RAS*, *K-RAS*, and *N-RAS*) are implicated in thyroid tumor formation from follicular cells, including 20% to 40% of benign follicular adenomas, 40% to 50% of FTC (including 15% to 20% of oxyphilic variants), 10% to 20% of PTC (almost exclusively follicular variants of PTC), and 25% of PDTC.[19-22] The presence of a *RAS* mutation may portend more aggressive disease with worse outcomes, but this has not been extensively examined.[19,23] Each of these histologies has also been observed in transgenic mice expressing *RAS* mutations, although the presence of mutant RAS proteins alone is likely insufficient to cause tumor formation.[24,25]

PI3K/Akt Pathway

Inactivating germline mutations of the tumor suppressor gene *PTEN* cause Cowden syndrome, which carries a 50- to 70-fold increased risk for the development of DTC, especially FTC.[26,27] Loss of this tumor suppressor function leads to activation of PI3K, Akt, and mTOR, thus contributing to enhanced cell cycle progression, decreased apoptosis, and increased tumor proliferation. However, mutations in individual genes in this pathway are otherwise uncommonly reported as early oncogenic events. Instead, somatic mutations and/or overexpression of *PIK3CA* (which encodes the class I p110α catalytic subunit of PI3K), *AKT*, and *PTEN* are observed as frequent later events, especially in FTC, PDTC, and ATC.[20,28,29] Gene amplification as well as activating point mutations are observed in 10% to 20% of PDTC and 40% of ATC and can be found in tumors also bearing either *BRAF* or *RAS* mutations. *AKT* activation is also characteristic of the invasive fronts of aggressive DTC and has been reported to trigger increased cellular motility.[30]

PAX8/PPARγ

A chromosomal translocation, t(2:3) (q13;p25), results in the *PAX8/PPARγ* mutation, which couples the DNA binding domains of the thyroid transcription factor PAX8 with the entire coding sequence of the nuclear peroxisome

proliferator-activated receptor subtype γ1.[31] The actual mechanisms by which the encoded fusion protein contributes to thyroid tumorigenesis remain unclear. However, several critical pathways may be affected, including reduced expression of PTEN leading to increased activation of Akt, and a dominant-negative effect on the normal PPARγ transcription factor permitting enhanced cellular proliferation and reduction of apoptosis.[30,32] This mutation may be preferentially seen in younger patients with smaller tumors, which are generally better prognostic signs, but conversely are also seen in tumors with solid or nested histologies as well as with vascular invasion.[33]

Medullary Thyroid Carcinoma

RET

About 20% of MTC occurs in one of several familial syndromes: multiple endocrine neoplasia (MEN) 2A (which also includes parathyroid tumors and pheochromocytomas); MEN 2B (which also includes pheochromocytomas, intestinal ganglioneuromatosis, neuromas of the tongue and subconjunctiva, and Marfanoid habitus); and familial MTC (FMTC, which lacks the other clinical features of MEN 2A). Additional variants of MEN 2A have been reported that include cutaneous lichen amyloidosis and with Hirschsprung disease. Germline mutations in *RET* were identified as causative of these hereditary forms of MTC in two landmark 1993 studies.[34,35] Today, more than 99% of all cases of hereditary MTC can be attributed to one of numerous point mutations in *RET* that cause activation of the tyrosine kinase function of the RTK (Table 43-1). Given the ubiquitous nature of the mutation, it is not surprising that the disease begins with diffuse hyperplasia of all of the C cells, with eventual development of one or more malignant foci.

The most common germline mutation, a cysteine-to-arginine substitution at codon 634 (denoted C634R), accounts for at least half of all cases of MEN 2A and has also been extensively studied in vitro in the well-characterized TT cell line.[36] This mutation is found in the cysteine-rich extracellular domain of RET, a region responsible for ligand-dependent dimerization. However, in the setting of the C634R mutation, RET is capable of ligand-independent dimerization, leading to autophosphorylation of the intracellular tyrosine residues that are responsible for interaction with downstream signaling pathways. In contrast, a methionine-to-threonine substitution at codon 918 (denoted M918T) is associated with the more aggressive phenotype of MEN 2B. The M918T mutation occurs in the intracellular domain of RET, changing the conformation of the tyrosine kinase domain and allowing marked enhancement of autophosphorylation in the absence of dimerization. In addition, allelic imbalance, due to either increased copy number of the mutant *RET* allele or deletion of part or all of the wild-type allele, has been reported in several cases of MEN 2A as well as the TT cell line itself.

Table 43-1 Most Common Mutations of the *RET* Gene Causing Hereditary Medullary Thyroid Carcinoma

Exon	Codon	Clinical Syndrome	Approximate Frequency
10	609	MEN 2A, FMTC, HD	≤1%
10	611	MEN 2A	2-3%
10	618	MEN 2A, FMTC, HD	3-5%
10	620	MEN 2A, FMTC, HD	6-8%
11	630	MEN 2A, FMTC	≤0.1%
11	634	MEN 2A (±CLA)	80%-90%
13	768	FMTC	≤1%
13	790	MEN 2A, FMTC	≤0.1%
13	791	FMTC	≤0.1%
14	804	FMTC	≤0.1%
15	891	MEN 2A, FMTC	≤1%
16	918	MEN 2B	10%-20%

From Hu MI, Jimenez C, Cote G, et al. Medullary thyroid carcinoma. In: Braverman LE, Cooper DS, eds. *Werner & Ingbar's The Thyroid: A Fundamental and Clinical Text*. 10th ed. Philadelphia, Pa: Wolters Kluwer; 2013:744-764.
CLA, Cutaneous lichen amyloidosis; *FMTC*, familial medullary thyroid carcinoma; *HD*, Hirschsprung disease; *MEN 2A*, multiple endocrine neoplasia type 2A; *MEN 2B*, multiple endocrine neoplasia type 2B.

Sporadic MTC, on the other hand, is not associated with germline changes in *RET*, but nonetheless, somatic *RET* mutations have been commonly reported in 25% to 50% of sporadic MTC cases. In this instance, the most frequent somatic mutation is the M918T alteration, but numerous other codon changes have also been observed, including selected deletions as well as point mutations. Of note, about 6% to 7% of patients with clinically sporadic MTC are found to carry germline mutations diagnostic of hereditary forms of the disease despite the absence of a positive family history, thus leading to the consensus recommendation to recommend *RET* germline testing for all newly diagnosed cases of apparently sporadic MTC.[37,38]

Extensive genotype:phenotype correlations have been established in the two decades since *RET* was identified as causing MTC. In addition to identifying specific clinical syndromes associated with each mutation, these analyses have also demonstrated that disease penetrance, typical age of development of C-cell hyperplasia and malignancy, and the aggressiveness of the malignancy vary in a manner that is based to a large degree on the individual mutation. Thus, the intracellular domain mutations, which tend to be associated

with the aggressive MTC characteristic of MEN 2B, are also found to cause aggressive sporadic MTC when they occur as somatic mutations. Patients who present with sporadic MTC associated with a somatic M918T mutation of *RET* have worse outcomes, including overall survival.[39] These genotype:phenotype correlations are also useful in determining the role and outcomes of genetic screening in hereditary disease. Recently published guidelines from the American Thyroid Association divide known *RET* germline mutations into four risk categories that guide earliest age for *RET* testing of potential familial carriers, earliest age for recommended first thyroid ultrasound and serum calcitonin testing to detect early presymptomatic evidence of disease, and role for potentially curative prophylactic thyroidectomy.[38] Using this type of approach, most young patients identified by prospective genetic screening as carriers for FMTC or MEN 2A can be cured with prophylactic thyroidectomy, although a small percentage remain with biochemical evidence of residual disease.[40]

RAS

Mutations of *RAS* have recently been recognized as common in sporadic MTC in the absence of documented *RET* mutations.[41,42] A wide range of frequency has been reported, however, between 10% and 80% of all *RET*–wild-type sporadic cases, using differing techniques for identifying *RAS* mutations. In the largest study, tumor samples from 108 sporadic disease patients without somatic *RET* mutations were subjected to *RAS* sequencing, yielding a frequency of 17% in that setting.[42] Of the three potential genotype combinations, patients who were (mutant)*RAS* (wt)*RET* were more likely to be disease free after a median follow-up of 5 years than those who were (wt)*RAS* (wt)*RET* or (wt)*RAS* (mutant)*RET*.

Other Molecular Mechanisms Active in Thyroid Carcinoma

As described earlier, tumor cells in DTC often express or overexpress cell surface RTKs for a variety of circulating growth factors, including VEGFR, FGFR, EGFR, PDGFRβ, IGFR, and MET. In addition, MTC cells also can contain similar cell surface RTKs for growth factors, including EGFR, MET, and FGFR. Overall, their roles appear to enhance the proliferative effects of mutated RTKs and intracellular signaling kinases, but in certain settings they may have critical functions. For example, studies of cancer stem cells, such as those derived from the MTC cell line MZ-CRC-1 that contains the M918T *RET* mutation, demonstrate the dependence on FGFR in the presence of RET knockdown for continued sphere formation and stem-cell proliferation.[43]

Of clear importance, however, is the role of growth factors secreted by thyroid tumor cells that interact with the neighboring stromal cells. This is particularly relevant for angiogenesis, by which tumor cells stimulate growth of vascular structures for supply of nutrition and oxygen as well as a conduit for distant metastasis. Cells from both DTC and MTC actively secrete various VEGF isoforms under conditions of limited oxygen, particularly VEGF-A, which interacts with VEGFR on neighboring vascular endothelial cells.[44-47] Similarly, cells from both DTC and MTC generate hepatocyte growth factor and FGF to stimulate MET and FGF receptors, respectively, on neighboring cells, an important pathway for stimulating angiogenesis in the absence of VEGFR activity.[48-50]

The TSH receptor has an indispensable role to stimulate thyrocyte proliferation normally. In neoplastic cells, the receptor is generally expressed and functional in DTC and PDTC, but usually absent in ATC. Short-term increases in the level of TSH can stimulate malignant cells with a functioning receptor, as evidenced by increases in differentiated functions such as thyroglobulin production and radioiodine incorporation, and tumor proliferation is similarly observed from chronic exposure to high levels of TSH.[51] There has also been recent evidence to suggest that TSH is necessary for BRAF-induced thyroid carcinogenesis.[52]

The tumor suppressor gene *TP53* is frequently mutated in advanced thyroid cancers. Point mutations that inactivate the suppressor protein are often seen in PDTC and ATC but are not seen in DTC.[53,54] Reexpression of normal p53 protein restores differentiated function in vivo.[55] Combining *TP53* mutation with other genetic lesions in animal models can reproduce the phenotype of ATC.[56,57]

The Wnt/β-catenin signaling pathway is often activated in advanced thyroid cancers, but recent data suggest a possible early role as well. Mutations in the scaffold proteins APC and Axin, along with β-catenin itself, have been reported in a majority of PDTC and ATC tumors, associated with increased tumor proliferation and loss of tumor differentiation.[58] In RET/PTC-mutant PTC, increased β-catenin localized to the nucleus has been observed as a result of posttranslational modification and protein stabilization.[59] Patients with familial adenomatosis and Gardner's syndrome, associated with mutations in the *APC* gene, have high risk for development of PTC, particularly an aggressive cribriform-morular variant.[60] In cases that have been examined, the presence of a germline *APC* mutation is associated with very high expression of β-catenin in the PTC cells along with frequent mutations in the gene as well as in *RET/PTC*.[61]

Therapeutic Targeting

The development of tyrosine kinase inhibitors (TKIs) that target many of the key oncogenes and other molecular abnormalities in thyroid cancer has led to the investigation of many agents in treatment of patients with advanced and metastatic disease.[62] Initial efforts focused on drugs that could inhibit activated RET kinase, such as vandetanib.[63] In this early study, a tyrosine kinase inhibitor that primarily inhibited VEGFR and EGFR was shown to block autophosphorylation of M918T and RET/PTC3, to prevent growth of cell lines with *RET/PTC1* mutations, and to inhibit growth of tumors after injection of fibroblasts transformed with the *RET/PTC3* gene. Based on findings like these, numerous multitargeted TKIs that can inhibit both RET and VEGFR (as well as other kinases) have been studied in the laboratory and in clinical trials. For example, vandetanib and cabozantinib (which also inhibits MET) significantly improve progression-free survival in patients with metastatic MTC.[64,65] Both drugs appear to be slightly more effective in patients whose tumors have *RET* mutations, but remain beneficial even in the absence of the targeted mutation. It remains to be determined whether these agents primarily work by targeting mutant RET kinase, through inhibition of angiogenesis through VEGFR (and MET for cabozantinib), or other mechanisms such as inhibiting the normal function of wild-type RET.

In DTC, initial interest in RET inhibitors waned when it was recognized that *RET/PTC* mutations are uncommon in advanced and metastatic disease. Instead, focus has been placed on targeting VEGFR-mediated angiogenesis with multikinase inhibitors such as motesanib, sorafenib, and sunitinib.[66-69] More recently, the availability of highly selective inhibitors of individual kinases in the MAPK pathway such as vemurafenib, dabrafenib, and selumetinib, has enabled trials evaluating oncogene targeting. Phase I experience with the two BRAF inhibitors, vemurafenib and dabrafenib, suggests that about one third of patients with BRAF-mutant PTC may respond to therapy[70,71]; phase II studies are under way. Selumetinib, an MEK inhibitor, has been studied in a fascinating pilot trial of 20 patients with progressive, radioiodine-refractory PTC, in which a 5-week course of selumetinib therapy followed immediately by radioiodine scanning induced restoration of enough radioiodine uptake and retention to permit high-dose radioiodine therapy to be subsequently administered to 7 of the patients; partial responses were observed in 5 patients who received the radioiodine therapy.[72]

Moving beyond inhibitors that target single pathways or kinases, studies are now under way to evaluate rational combinations of agents. For example, preclinical studies suggest that simultaneous inhibition of both MAPK and PI3K pathway signaling in DTC and MTC may be more effective than inhibiting either pathway individually, providing the rationale for combining drugs such as sorafenib and everolimus. Similarly, inhibition of both BRAF and MEK in melanoma yielded a potentially more effective regimen with fewer side effects than use of a BRAF inhibitor alone, and this approach is also being tested in BRAF-mutant PTC.[73]

Future Directions

The molecular abnormalities described here represent a broad effort during the past 20 years to understand the fundamental pathophysiology of thyroid cancer. Whereas early oncogenic events have been identified that probably account for the majority of these tumors, further study is needed to identify the incipient events in the remaining tumors. Just as critical is the need for a more comprehensive understanding of the steps that lead to progression, invasion, metastasis, and occasional dedifferentiation. An integrated framework will be required that merges knowledge of DNA mutations with understanding of the role of epigenetic alterations, changes in miRNA regulation of gene expression, and other fundamental processes that contribute to the malignant phenotype. Clinical trials of therapies to reverse genetic changes and alter complex signaling abnormalities will need to be informed by comprehensive, individualized tumor profiling that will facilitate the selection of the correct combination of therapies for each individual patient, including the identification of patients with sufficiently indolent tumors that no therapy will ever be required.

Key References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
4. Sherman SI. Thyroid carcinoma. *Lancet*. 2003;361:501-511.
7. Croyle M, Akeno N, Knauf JA, et al. RET/PTC-induced cell growth is mediated in part by epidermal growth factor receptor (EGFR) activation: evidence for molecular and functional interactions between RET and EGFR. *Cancer Res*. 2008;68:4183-4191.
9. Pierotti MA, Greco A. Oncogenic rearrangements of the NTRK1/NGF receptor. *Cancer Lett*. 2006;232:90-98.
11. Hamatani K, Eguchi H, Ito R, et al. RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. *Cancer Res*. 2008;68:7176-7182.
13. Tufano RP, Teixeira GV, Bishop J, et al. BRAF mutation in papillary thyroid cancer and its value in tailoring initial treatment: a systematic review and meta-analysis. *Medicine (Baltimore)*. 2012;91:274-286.

14. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell*. 2004;116:855-867.
15. Nikiforov YE, Ohori NP, Hodak SP, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab*. 2011;96:3390-3397.
16. Elisei R, Ugolini C, Viola D, et al. BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. *J Clin Endocrinol Metab*. 2008;93:3943-3949.
17. Chakravarty D, Santos E, Ryder M, et al. Small-molecule MAPK inhibitors restore radioi47odine incorporation in mouse thyroid cancers with conditional BRAF activation. *J Clin Invest*. 2011;121:4700-4711.
18. Hou P, Liu D, Xing M. Genome-wide alterations in gene methylation by the BRAF V600E mutation in papillary thyroid cancer cells. *Endocr Relat Cancer*. 2011;18:687-697.
19. Volante M, Rapa I, Gandhi M, et al. RAS mutations are the predominant molecular alteration in poorly differentiated thyroid carcinomas and bear prognostic impact. *J Clin Endocrinol Metab*. 2009;94:4735-4741.
20. Ricarte-Filho JC, Ryder M, Chitale DA, et al. Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. *Cancer Res*. 2009;69:4885-4893.
22. Maximo V, Lima J, Prazeres H, et al. The biology and the genetics of Hurthle cell tumors of the thyroid. *Endocr Relat Cancer*. 2012;19:R131-R147.
23. Garcia-Rostan G, Zhao H, Camp RL, et al. RAS mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. *J Clin Oncol*. 2003;21:3226-3235.
24. Vitagliano D, Portella G, Troncone G, et al. Thyroid targeting of the N-ras(Gln61Lys) oncogene in transgenic mice results in follicular tumors that progress to poorly differentiated carcinomas. *Oncogene*. 2006;25:5467-5474.
25. Miller KA, Yeager N, Baker K, et al. Oncogenic Kras requires simultaneous PI3K signaling to induce ERK activation and transform thyroid epithelial cells in vivo. *Cancer Res*. 2009;69:3689-3694.
26. Ngeow J, Mester J, Rybicki LA, et al. Incidence and clinical characteristics of thyroid cancer in prospective series of individuals with Cowden and Cowden-like syndrome characterized by germline PTEN, SDH, or KLLN alterations. *J Clin Endocrinol Metab*. 2011;96:E2063-E2071.
27. Tan MH, Mester JL, Ngeow J, et al. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res*. 2012;18:400-407.
28. Santarpia L, El-Naggar AK, Cote GJ, et al. Phosphatidylinositol 3-kinase/Akt and RAS/RAF-mitogen-activated protein kinase pathway mutations in anaplastic thyroid cancer. *J Clin Endocrinol Metab*. 2008;93:278-284.
29. Saji M, Ringel MD. The PI3K-Akt-mTOR pathway in initiation and progression of thyroid tumors. *Mol Cell Endocrinol*. 2010;321:20-28.
30. Vasko V, Saji M, Hardy E, et al. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. *J Med Genet*. 2004;41:161-170.
32. Reddi HV, McIver B, Grebe SK, et al. The paired box-8/peroxisome proliferator-activated receptor-gamma oncogene in thyroid tumorigenesis. *Endocrinology*. 2007;148:932-935.
38. Kloos RT, Eng C, Evans DB, et al. Medullary thyroid cancer: management guidelines of the American Thyroid Association. *Thyroid*. 2009;19:565-612.
39. Elisei R, Cosci B, Romei C, et al. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab*. 2008;93:682-687.
40. Skinner MA, Moley JA, Dilley WG, et al. Prophylactic thyroidectomy in multiple endocrine neoplasia type 2A. *N Engl J Med*. 2005;353:1105-1113.
41. Boichard A, Croux L, Al Ghuzlan A, et al. Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid carcinomas and extend to a previously unidentified exon. *J Clin Endocrinol Metab*. 2012;97:E2031-E2035.
42. Ciampi R, Mian C, Fugazzola L, et al. Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. *Thyroid*. 2013;23:50-57.
43. Zhu W, Hai T, Ye L, et al. Medullary thyroid carcinoma cell lines contain a self-renewing CD133+ population that is dependent on Ret proto-oncogene activity. *J Clin Endocrinol Metab*. 2010;95:439-444.
44. Capp C, Wajner SM, Siqueira DR, et al. Increased expression of vascular endothelial growth factor and its receptors, VEGFR-1 and VEGFR-2, in medullary thyroid carcinoma. *Thyroid*. 2010;20:863-871.
45. Jo YS, Li S, Song JH, et al. Influence of the BRAF V600E mutation on expression of vascular endothelial growth factor in papillary thyroid cancer. *J Clin Endocrinol Metab*. 2006;91:3667-3670.
46. Vieira JM, Santos SCR, Espadinha C, et al. Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop. *Eur J Endocrinol*. 2005;153:701-709.
47. Rodriguez-Antona C, Pallares J, Montero-Conde C, et al. Overexpression and activation of EGFR and VEGFR2 in medullary thyroid carcinomas is related to metastasis. *Endocr Relat Cancer*. 2010;17:7-16.
52. Franco AT, Malaguarnera R, Refetoff S, et al. Thyrotrophin receptor signaling dependence of Braf-induced thyroid tumor initiation in mice. *Proc Natl Acad Sci U S A*. 2011;108:1615-1620.
56. Antico Arciuch VG, Russo MA, Dima M, et al. Thyrocyte-specific inactivation of p53 and Pten results in anaplastic thyroid carcinomas faithfully recapitulating human tumors. *Oncotarget*. 2011;2:1109-1126.
59. Castellone MD, De Falco V, Rao DM, et al. The beta-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. *Cancer Res*. 2009;69:1867-1876.
60. Groen EJ, Roos A, Muntinghe FL, et al. Extra-intestinal manifestations of familial adenomatous polyposis. *Ann Surg Oncol*. 2008;15:2439-2450.
61. Sastre-Perona A, Santisteban P. Role of the Wnt pathway in thyroid cancer. *Front Endocrinol (Lausanne)*. 2012;3:31.
62. Sherman SI. Targeted therapies for thyroid tumors. *Mod Pathol*. 2011;24(suppl 2):S44-S52.
64. Wells Jr SA, Robinson BG, Gagel RF, et al. Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *J Clin Oncol*. 2012;30(2):134-141.
65. Schoffski P, Elisei R, Muller S, et al. An international, double-blind, randomized, placebo-controlled phase III trial (EXAM) of cabozantinib (XL184) in medullary thyroid carcinoma (MTC) patients (pts) with documented RECIST progression at baseline. *J Clin Oncol*. 2012;30(suppl):5508.
66. Sherman SI, Wirth LJ, Droz JP, et al. Motesanib diphosphate in progressive differentiated thyroid cancer. *N Engl J Med*. 2008;359:31-42.
67. Brose MS, Nutting CM, Sherman SI, et al. Rationale and design of DECISION: a double-blind, randomized, placebo-controlled phase III trial evaluating the efficacy and safety of sorafenib in patients with locally advanced or metastatic radioactive iodine (RAI)-refractory, differentiated thyroid cancer. *BMC Cancer*. 2011;11:349.
68. Kloos RT, Ringel MD, Knopp MV, et al. Phase II trial of sorafenib in metastatic thyroid cancer. *J Clin Oncol*. 2009;27:1675-1684.
69. Carr LL, Mankoff DA, Goulart BH, et al. Phase II study of daily sunitinib in FDG-PET-positive, iodine-refractory differentiated thyroid cancer and metastatic medullary carcinoma of the thyroid with functional imaging correlation. *Clin Cancer Res*. 2010;16:5260-5268.
70. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
71. Falchook GS, Long GV, Kurzrock R, et al. Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet*. 2012;379:1893-1901.
72. Ho AL, Leboeuf R, Grewal RK, et al. Reacquisition of RAI uptake in RAI-refractory metastatic thyroid cancers by pretreatment with the MEK inhibitor selumetinib. *J Clin Oncol*. 2012;30(suppl):5509.
73. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367:1694-1703.

This page intentionally left blank

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012;62:10-29.
3. Altekruse SF, Kosary CL, Krapcho M, et al. *SEER Cancer Statistics Review, 1975-2007*. Bethesda, MD: National Cancer Institute; 2010.
4. Sherman SI. Thyroid carcinoma. *Lancet*. 2003;361:501-511.
5. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci*. 2002;3:383-394.
6. Fusco A, Santoro M, Grieco M, et al. RET/PTC activation in human thyroid carcinomas. *J Endocrinol Invest*. 1995;18:127-129.
7. Croyle M, Akeno N, Knauf JA, et al. RET/PTC-induced cell growth is mediated in part by epidermal growth factor receptor (EGFR) activation: evidence for molecular and functional interactions between RET and EGFR. *Cancer Res*. 2008;68:4183-4191.
8. Jhiang SM, Cho JY, Furminger TL, et al. Thyroid carcinomas in RET/PTC transgenic mice. *Recent Results Cancer Res*. 1998;154:265-270.
9. Pierotti MA, Greco A. Oncogenic rearrangements of the NTRK1/NGF receptor. *Cancer Lett*. 2006;232:90-98.
10. Rabes HM. Gene rearrangements in radiation-induced thyroid carcinogenesis. *Med Pediatr Oncol*. 2001;36:574-582.
11. Hamatani K, Eguchi H, Ito R, et al. RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. *Cancer Res*. 2008;68:7176-7182.
12. Nikiforova MN, Stringer JR, Blough R, et al. Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. *Science*. 2000;290:138-141.
13. Tufano RP, Teixeira GV, Bishop J, et al. BRAF mutation in papillary thyroid cancer and its value in tailoring initial treatment: a systematic review and meta-analysis. *Medicine (Baltimore)*. 2012;91:274-286.
14. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell*. 2004;116:855-867.
15. Nikiforov YE, Ohori NP, Hodak SP, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab*. 2011;96:3390-3397.
16. Elisei R, Ugolini C, Viola D, et al. BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. *J Clin Endocrinol Metab*. 2008;93:3943-3949.
17. Chakravarty D, Santos E, Ryder M, et al. Small-molecule MAPK inhibitors restore radioi47odine incorporation in mouse thyroid cancers with conditional BRAF activation. *J Clin Invest*. 2011;121:4700-4711.
18. Hou P, Liu D, Xing M. Genome-wide alterations in gene methylation by the BRAF V600E mutation in papillary thyroid cancer cells. *Endocr Relat Cancer*. 2011;18:687-697.
19. Volante M, Rapa I, Gandhi M, et al. RAS mutations are the predominant molecular alteration in poorly differentiated thyroid carcinomas and bear prognostic impact. *J Clin Endocrinol Metab*. 2009;94:4735-4741.
20. Ricarte-Filho JC, Ryder M, Chitale DA, et al. Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. *Cancer Res*. 2009;69:4885-4893.
21. Lemoine NR, Mayall ES, Wyllie FS, et al. High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene*. 1989;4:159-164.
22. Maximo V, Lima J, Prazeres H, et al. The biology and the genetics of Hurthle cell tumors of the thyroid. *Endocr Relat Cancer*. 2012;19:R131-R147.
23. Garcia-Rostan G, Zhao H, Camp RL, et al. RAS mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. *J Clin Oncol*. 2003;21:3226-3235.
24. Vitagliano D, Portella G, Troncone G, et al. Thyroid targeting of the N-ras(Gln61Lys) oncogene in transgenic mice results in follicular tumors that progress to poorly differentiated carcinomas. *Oncogene*. 2006;25:5467-5474.
25. Miller KA, Yeager N, Baker K, et al. Oncogenic Kras requires simultaneous PI3K signaling to induce ERK activation and transform thyroid epithelial cells in vivo. *Cancer Res*. 2009;69:3689-3694.
26. Ngeow J, Mester J, Rybicki LA, et al. Incidence and clinical characteristics of thyroid cancer in prospective series of individuals with Cowden and Cowden-like syndrome characterized by germline PTEN, SDH, or KLLN alterations. *J Clin Endocrinol Metab*. 2011;96:E2063-E2071.
27. Tan MH, Mester JL, Ngeow J, et al. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res*. 2012;18:400-407.
28. Santarpia L, El-Naggar AK, Cote GJ, et al. Phosphatidylinositol 3-kinase/Akt and RAS/RAF-mitogen-activated protein kinase pathway mutations in anaplastic thyroid cancer. *J Clin Endocrinol Metab*. 2008;93:278-284.
29. Saji M, Ringel MD. The PI3K-Akt-mTOR pathway in initiation and progression of thyroid tumors. *Mol Cell Endocrinol*. 2010;321:20-28.
30. Vasko V, Saji M, Hardy E, et al. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. *J Med Genet*. 2004;41:161-170.
31. Kroll TG, Sarraf P, Pecciarini L, et al. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma. *Science*. 2000;289:1357-1360.
32. Reddi HV, McIver B, Grebe SK, et al. The paired box-8/peroxisome proliferator-activated receptor-gamma oncogene in thyroid tumorigenesis. *Endocrinology*. 2007;148:932-935.
33. Nikiforova MN, Biddinger PW, Caudill CM, et al. PAX8-PPARgamma rearrangement in thyroid tumors: RT-PCR and immunohistochemical analyses. *Am J Surg Pathol*. 2002;26:1016-1023.
34. Mulligan LM, Kwok JB, Healey CS, et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature*. 1993;363:458-460.
35. Donis-Keller H, Dou S, Chi D, et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet*. 1993;2:851-856.
36. Santoro M, Carlomagno F, Romano A, et al. Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science*. 1995;267:381-383.
37. Wohllk N, Cote GJ, Bugalho MMJ, et al. Relevance of RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. *J Clin Endocrinol Metab*. 1996;81:3740-3745.
38. Kloos RT, Eng C, Evans DB, et al. Medullary thyroid cancer: management guidelines of the American Thyroid Association. *Thyroid*. 2009;19:565-612.
39. Elisei R, Cosci B, Romei C, et al. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab*. 2008;93:682-687.
40. Skinner MA, Moley JA, Dilley WG, et al. Prophylactic thyroidectomy in multiple endocrine neoplasia type 2A. *N Engl J Med*. 2005;353:1105-1113.
41. Boichard A, Croux L, Al Ghuzlan A, et al. Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid carcinomas and extend to a previously unidentified exon. *J Clin Endocrinol Metab*. 2012;97:E2031-E2035.
42. Ciampi R, Mian C, Fugazzola L, et al. Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. *Thyroid*. 2013;23:50-57.
43. Zhu W, Hai T, Ye L, et al. Medullary thyroid carcinoma cell lines contain a self-renewing CD133+ population that is dependent on Ret proto-oncogene activity. *J Clin Endocrinol Metab*. 2010;95:439-444.
44. Capp C, Wajner SM, Siqueira DR, et al. Increased expression of vascular endothelial growth factor and its receptors, VEGFR-1 and VEGFR-2, in medullary thyroid carcinoma. *Thyroid*. 2010;20:863-871.
45. Jo YS, Li S, Song JH, et al. Influence of the BRAF V600E mutation on expression of vascular endothelial growth factor in papillary thyroid cancer. *J Clin Endocrinol Metab*. 2006;91:3667-3670.
46. Vieira JM, Santos SCR, Espadinha C, et al. Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop. *Eur J Endocrinol*. 2005;153:701-709.
47. Rodriguez-Antona C, Pallares J, Montero-Conde C, et al. Overexpression and activation of EGFR and VEGFR2 in medullary thyroid carcinomas is related to metastasis. *Endocr Relat Cancer*. 2010;17:7-16.
48. Papotti M, Olivero M, Volante M, et al. Expression of hepatocyte growth factor (HGF) and its receptor (MET) in medullary carcinoma of the thyroid. *Endocr Pathol*. 2000;11:19-30.

49. Ramirez R, Hsu D, Patel A, et al. Over-expression of hepatocyte growth factor/scatter factor (HGF/SF) and the HGF/SF receptor (cMET) are associated with a high risk of metastasis and recurrence for children and young adults with papillary thyroid carcinoma. *Clin Endocrinol (Oxf)*. 2000;53:635-644.
50. Eggo MC, Hopkins JM, Franklyn JA, et al. Expression of fibroblast growth factors in thyroid cancer. *J Clin Endocrinol Metab*. 1995;80:1006-1011.
51. Hoelting T, Tezelman S, Siperstein AE, et al. Biphasic effects of thyrotropin on invasion and growth of papillary and follicular thyroid cancer in vitro. *Thyroid*. 1995;5:35-40.
52. Franco AT, Malaguarnera R, Refetoff S, et al. Thyrotrophin receptor signaling dependence of Braf-induced thyroid tumor initiation in mice. *Proc Natl Acad Sci U S A*. 2011;108:1615-1620.
53. Donghi R, Longoni A, Pilotti S, et al. Gene p53 mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. *J Clin Invest*. 1993;91:1753-1760.
54. Ito T, Seyama T, Mizuno T, et al. Unique association of p53 mutations with undifferentiated but not with differentiated carcinomas of the thyroid gland. *Cancer Res*. 1992;52:1369-1371.
55. Moretti F, Farsetti A, Soddu S, et al. p53 re-expression inhibits proliferation and restores differentiation of human thyroid anaplastic carcinoma cells. *Oncogene*. 1997;14:729-740.
56. Antico Arciuch VG, Russo MA, Dima M, et al. Thyrocyte-specific inactivation of p53 and Pten results in anaplastic thyroid carcinomas faithfully recapitulating human tumors. *Oncotarget*. 2011;2:1109-1126.
57. La Perle KM, Jhiang SM, Capen CC. Loss of p53 promotes anaplasia and local invasion in ret/PTC1-induced thyroid carcinomas. *Am J Pathol*. 2000;157:671-677.
58. Garcia-Rostan G, Camp RL, Herrero A, et al. Beta-catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. *Am J Pathol*. 2001;158:987-996.
59. Castellone MD, De Falco V, Rao DM, et al. The beta-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. *Cancer Res*. 2009;69:1867-1876.
60. Groen EJ, Roos A, Muntinghe FL, et al. Extra-intestinal manifestations of familial adenomatous polyposis. *Ann Surg Oncol*. 2008;15:2439-2450.
61. Sastre-Perona A, Santisteban P. Role of the Wnt pathway in thyroid cancer. *Front Endocrinol (Lausanne)*. 2012;3:31.
62. Sherman SI. Targeted therapies for thyroid tumors. *Mod Pathol*. 2011;24(suppl 2):S44-S52.
63. Carlomagno F, Vitagliano D, Guida T, et al. ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. *Cancer Res*. 2002;62:7284-7290.
64. Wells Jr SA, Robinson BG, Gagel RF, et al. Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *J Clin Oncol*. 2012;30(2):134-141.
65. Schoffski P, Elisei R, Muller S, et al. An international, double-blind, randomized, placebo-controlled phase III trial (EXAM) of cabozantinib (XL184) in medullary thyroid carcinoma (MTC) patients (pts) with documented RECIST progression at baseline. *J Clin Oncol*. 2012;30(suppl):5508.
66. Sherman SI, Wirth LJ, Droz JP, et al. Motesanib diphosphate in progressive differentiated thyroid cancer. *N Engl J Med*. 2008;359:31-42.
67. Brose MS, Nutting CM, Sherman SI, et al. Rationale and design of DECISION: a double-blind, randomized, placebo-controlled phase III trial evaluating the efficacy and safety of sorafenib in patients with locally advanced or metastatic radioactive iodine (RAI)-refractory, differentiated thyroid cancer. *BMC Cancer*. 2011;11:349.
68. Kloos RT, Ringel MD, Knopp MV, et al. Phase II trial of sorafenib in metastatic thyroid cancer. *J Clin Oncol*. 2009;27:1675-1684.
69. Carr LL, Mankoff DA, Goulart BH, et al. Phase II study of daily sunitinib in FDG-PET-positive, iodine-refractory differentiated thyroid cancer and metastatic medullary carcinoma of the thyroid with functional imaging correlation. *Clin Cancer Res*. 2010;16:5260-5268.
70. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
71. Falchook GS, Long GV, Kurzrock R, et al. Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet*. 2012;379:1893-1901.
72. Ho AL, Leboeuf R, Grewal RK, et al. Reacquisition of RAI uptake in RAI-refractory metastatic thyroid cancers by pretreatment with the MEK inhibitor selumetinib. *J Clin Oncol*. 2012;30(suppl):5509.
73. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367:1694-1703.

Julie D. R. Reimann and Christopher D. M. Fletcher

44

Soft Tissue Sarcomas

Introduction

Soft tissue sarcomas are a heterogeneous group of neoplasms arising in mesenchymal tissue. There are more than 35 histologic subtypes, often associated with distinctive clinicopathologic features. Based on advances in our knowledge of the molecular pathogenesis of these tumors over the past decade, sarcomas can be subdivided into those with recurrent, usually quite simple genetic alterations (approximately one third of sarcomas; Table 44-1), and those with nonrecurrent genetic alterations.[1,2] Examples of the latter group include leiomyosarcomas, malignant peripheral nerve sheath tumors, and unclassified high-grade pleomorphic and spindle-cell sarcomas (often formerly known as "malignant fibrous histiocytomas"), all of which lack specific recurrent genetic aberrations and instead exhibit complex karyotypes including multiple chromosomal deletions, losses, and gains. These last sarcomas frequently have alterations in the *p53* tumor suppressor pathway and a clinically aggressive course.[3] Recurrent translocations in the former group are typically the only cytogenetic alteration present and often involve transcription factors (e.g., *FOXO1A*, *WT1*, *PAX3*, *TFE3*, *FLI1*). This chapter focuses on three tumors with well-characterized genetic alterations—synovial sarcoma, well-differentiated liposarcoma, and gastrointestinal stromal tumor—as prototypical examples of translocation-associated, gene amplification–associated, and oncogenic mutation–associated sarcomas, respectively. The identification of specific genomic alterations in these and other sarcomas is leading to both enhanced prognostication as well as identification of potential therapeutic targets.

Synovial Sarcoma

Clinical Description and Pathology

Synovial sarcoma (SS) accounts for approximately 8% to 10% of all sarcomas; it is the most common sarcoma of young adults.[4] The peak incidence is between ages 15 and 40, and the majority occurs before age 50,[5] with males slightly more affected than females. SS most commonly arises in the extremities (90%), although almost any other site can be affected. It typically presents as a slowly growing, painful mass, with calcifications often being present radiologically.[6] It is an aggressive sarcoma, with a 5-year mortality rate ranging from 25% to 76%.[7-9] Up to 50% of SS recur, usually within 2 years of initial diagnosis. The most common location for metastases is the lungs, and the most important predictor of metastasis is tumor size, with size greater than 5 cm correlating with a significantly increased metastatic risk.[8] Advanced age and advanced stage at presentation, as well as poorly differentiated histology, also correlate with a worse outcome.

Histologically, SS is a mesenchymal neoplasm displaying varying degrees of epithelial differentiation and bearing no biologic relationship to synovial tissue. The tumor was originally designated SS because its tendency to occur near articular surfaces and the frequent presence of an epithelioid component initially suggested origin from synovial tissue. However, ultrastructural, immunohistochemical, and genetic analyses have demonstrated true epithelial and mesenchymal (but not synovial) differentiation.[4,10] Moreover, a mouse model for SS implicates skeletal muscle (particularly myoblasts) as a potential tumor source.[10] In this mouse model, tumors occurred only within a background of myoblasts expressing myogenic regulatory factor myf5 and not in more differentiated myoid lineages.[10]

SS is divided into two principal morphologic subtypes, biphasic and monophasic, based on the presence or absence of glandular epithelial differentiation. The biphasic variant is characterized by spindle-cell areas intermingled with an epithelial component, often forming glands, tubules or nests (Figure 44-1, *A*). The epithelial cells are usually larger, with paler nuclei and more abundant cytoplasm than the spindle-cell component. The epithelial component can be very focal, making it difficult to detect unless highlighted by keratin stains. The spindle-cell areas are composed of closely packed cells with hyperchromatic nuclei and scant cytoplasm,

Table 44-1 Soft Tissue Sarcomas with Recurrent Genetic Alterations

Tumor	Recurrent Genetic Abnormality	Genes Involved
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1:13)(p36;q14)	*PAX3-FOXO1A* *PAX7-FOXO1A*
Alveolar soft-part sarcoma	t(X;17)(p11;q25)	*TFE3-ASPL*
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	*CREB1-EWSR1* *ATF1-FUS*
Clear cell sarcoma	t(12;22)(q13;q12)	*ATF1-EWSR1* *CREB1-EWSR1*
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	*PDGFB-COL1A1*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*WT1-EWSR1*
Embryonal rhabdomyosarcoma	LOH at 11p15	*BWSCR1A* *IGF2*
Ewing sarcoma/peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q12;q12) t(2;22)(q33;q12)	*FLI-1-EWSR1* *ERG-EWSR1* *ETV1-EWSR1* *E1AF-EWSR1* *FEV-EWSR1*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21)	*NR4A3-EWSR1* *NR4A3-RBP56* *NR4A3-TCF12*
Gastrointestinal stromal sarcoma	Activating mutations	*c-kit* *PDGFRA*
Infantile fibrosarcoma	t(12:15)(p13;q25)	*ETV6-NTRK3*
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11) t(11;16)(p11;p11)	*CREB3L2-FUS* *CREB3L1-FUS*
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23) t(2;19)(p23;p13) t(2;17)(p23;q23) t(2;2)(p23;q13)	*ALK-TPM3* *ALK-TPM4* *ALK-CLTC* *ALK-RANBP2*
Malignant rhabdoid tumor	Deletion 22q11	*SMARCB1*
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12	*DDIT3-FUS* *DDIT3-EWSR1*
Synovial sarcoma	t(X;18)(p11;q11)	*SSX1-SYT* *SSX2-SYT* *SSX4-SYT*
Well-differentiated liposarcoma/atypical lipomatous tumor	12q14–15 amplification	*MDM2* *CDK4* *SAS* *HMGA2*

LOH, Loss of heterozygosity.

imparting an overlapping appearance, growing in sheets and/or fascicles. Keratins and other epithelial immunohistochemical markers are more abundant in the epithelial component; however, they (especially epithelial membrane antigen) are also expressed, albeit to a lesser extent, in the spindle-cell areas. The monophasic variant is composed entirely of the spindle-cell component and is the more common subtype (see Figure 44-1, *B*). Another characteristic hallmark of both biphasic and monophasic variants is the presence of branching vessels, termed *hemangiopericytoma-like*, as well as wiry stromal collagen. SS with areas of densely packed small round to spindled cells and a high mitotic rate (with or without necrosis) are designated poorly differentiated and, as mentioned earlier, have a worse prognosis.

Genetics and Molecular Pathogenesis

SS contain a specific translocation between chromosomes X and 18 [t(X;18);(p11.2;q11.2)].[4] This translocation results in fusion of the *SYT* gene on chromosome 18 with either *SSX1* or *SSX2* (or rarely *SSX4*) on chromosome X. The resulting fusion gene contains *SYT* gene minus the final 8 C-terminal amino acids and the C terminus of the *SSX* gene (see Figure 44-1, *C*).

In the literature to date, it appears that biphasic SS much more commonly carries the *SYT-SSX1* fusion, whereas monophasic SS shows either *SYT-SSX1* or *SYT-SSX2*.[11-13] This trend between fusion gene product and histologic type suggests the possibility that the *SYT-SSX1* fusion gene is more efficient at promoting epithelial differentiation than the *SYT-SSX2* fusion gene. However, some biphasic SS carry the *SYT-SSX2* rather than the *SYT-SSX1* transcript, so this reported association is by no means consistent.

Attempts have also been made to correlate fusion type with prognosis, although the results are conflicting. Several studies suggested that patients with the *SYT-SSX2* translocation have a better prognosis than those with the *SYT-SSX1* transcript;[13,14] others have found no statistically significant correlation between fusion type and prognosis.[12] All of these studies were retrospective, and none could control for differences in treatment or selection bias in follow-up data.[15] The proposed correlation of fusion gene type with prognosis therefore remains unconfirmed.

SYT is widely expressed during early murine embryogenesis and in the adult.[11] The gene encodes a protein with three possible SH2- and one possible SH3-binding domains (likely protein-protein interaction domains), a novel N-terminal domain (termed an *SNH domain*), and a C-terminal domain rich in glutamine, proline, glycine, and tyrosine (QPGY domain) similar to those found in the *EWSR1* gene and other transcriptional activators (see Figure 44-1, *C*). There is in vitro evidence that *SYT* can act as a transcriptional activator and *SYT* has been shown to bind to the *SWI/SNF* chromatin remodeling complex.[16] *SYT* also interacts with the p300 nuclear pore protein, and this interaction appears to promote cell-cell adhesion.[17] An *SYT* deletion mutant lacking the eight C-terminal amino acids (the most common *SYT* mutant found in the *SYT/SSX* fusion gene product) acts in a dominant negative fashion, preventing cell adhesion to an extracellular matrix.[17]

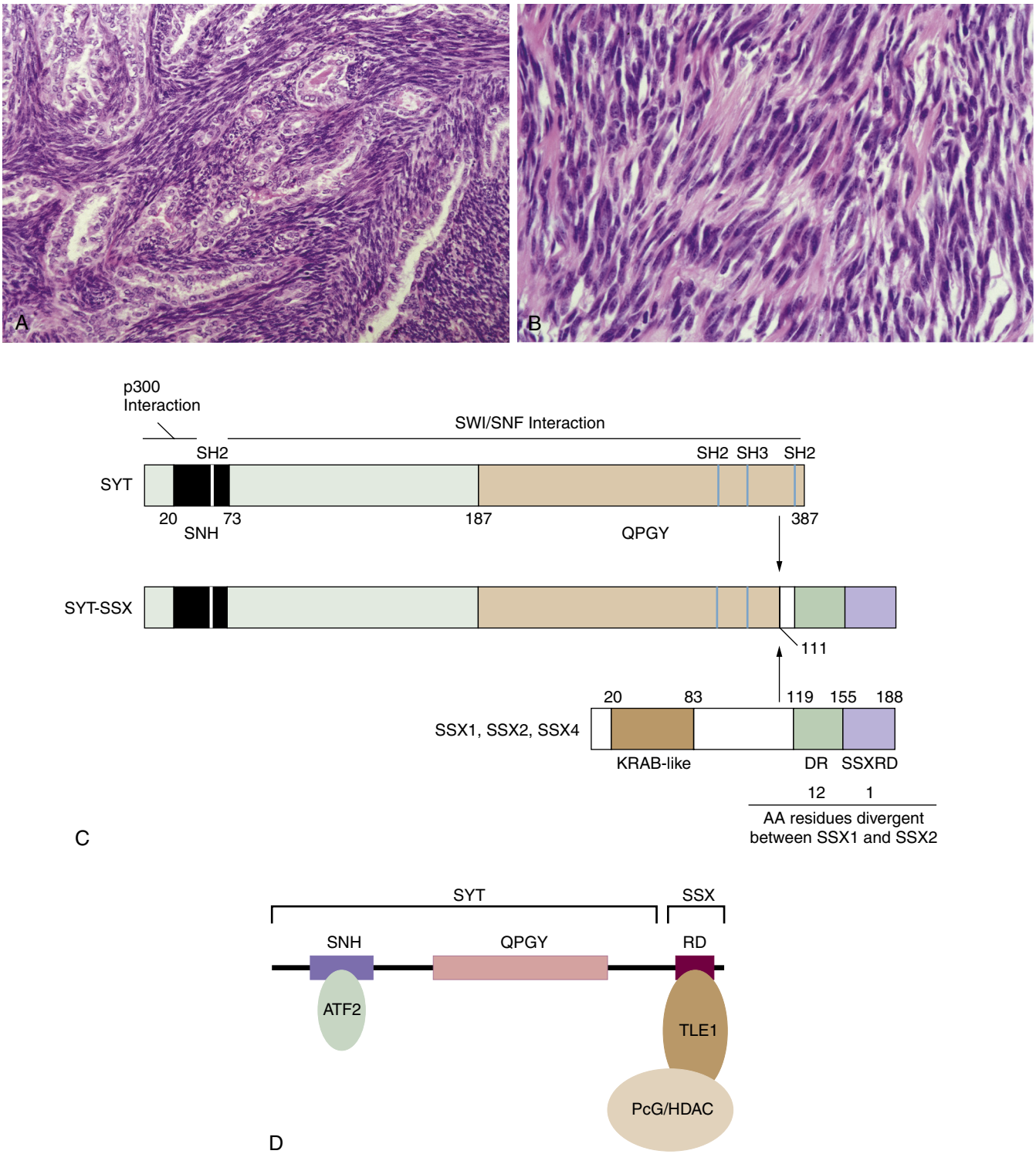


Figure 44-1 Synovial sarcoma **(A)** Biphasic synovial sarcoma, with glandular differentiation within a spindle cell background. **(B)** Monophasic synovial sarcoma, demonstrating the typical densely packed spindle cells growing in a fascicular pattern within a background of wiry stromal collagen. **(C)** Diagram of SYT, SSX, and the SYT-SSX fusion proteins. **(D)** Interaction of SYT-SSX with ATF2 and TLE1 leads recruitment of PcG/HDAC via TLE1. *AA,* Amino acids; *DR,* SSX divergent region; *KRAB,* Kruppel-associated box; *QPGY,* SYT glutamine, proline, glycine, and tyrosine-rich domain; *SNH,* SYT N-terminal domain; *SSXRD,* SSX repressor domain.

SSX1 and *SSX2* share significant homology (81% identity) to each other and belong to a gene family whose expression is predominantly restricted to germ cells and tumors.[11] The *SSX* protein contains an N-terminal domain similar to the Kruppel-associated box (KRAB) domain found in several transcriptional repressors, and an acidic C-terminal region (SSX-RD) that appears to be a novel transcriptional repressor domain[18] required for colocalization with polycomb group (PcG) proteins, which are important for maintaining transcriptional repression from one cell cycle to the next.[19]

The transforming capability of the *SYT-SSX1* fusion gene product has been demonstrated in vitro and in vivo.[20] The N-terminal region of *SYT* (which binds to the *SWI/SNF* chromatin remodeling complex) is required for the transforming properties of *SYT-SSX1*, suggesting a role for transcriptional regulation by *SYT-SSX1* via the *SWI/SNF* complex in SS tumorigenesis. Recently, proteins involved in the transcriptional regulatory activity of SYT-SSX have been identified, including activating transcription factor 2 (ATF2; a transcriptional activator), TLE1 (a transcriptional repressor), and histone deacetylases (HDAC).[21] TLE1 is strongly expressed in SS and is a sensitive diagnostic marker for SS.[22-24] Knockdown of both ATF2 and TLE1 in both human and mouse SS cell lines via small interfering RNA (siRNA) prevented tumor cell colony formation and inhibited cell growth,[21] supporting a role for these proteins in the oncogenic activity of SYT-SSX in SS. The association of SYT-SSX with ATF2 and TLE1 results in repression of ATF2 target genes via recruitment of HDAC/PcG by TLE1 (see Figure 44-1, *D*).

The role of the HDAC/PcG complex in the oncogenic effects of SYT-SSX provides a clinically relevant therapeutic target, namely, HDAC inhibitor targeted therapy. In preclinical studies, HDAC inhibitors have shown early success in SS growth inhibition and remain an area of significant interest in SS treatment.[21,25,26]

In addition to a potential role for HDAC inhibitors in SS targeted therapy, recent data have implicated a role for the phosphatidylinositol-3′-kinase (PI3K)/AKT signaling pathway in SS tumorigenesis,[27] as this signaling is frequently activated in SS. Inhibition of PI3K also decreased SS growth in vitro.[27] These data point to a potential role for PI3K inhibitors in SS therapeutic strategies.

Atypical Lipomatous Tumor/ Well-Differentiated Liposarcoma

Clinical Description and Pathology

Liposarcoma as a class is the most common malignant soft tissue neoplasm. Of the several distinct subtypes, atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL), a sarcoma of intermediate (locally aggressive) malignancy, is the most common.[28] It is a tumor of older adults, most often presenting in the sixth or seventh decades. Males and females are equally affected. ALT/WDL occurs most frequently in the extremities or retroperitoneum, followed by the paratesticular region, mediastinum, and head and neck. They tend to be deep-seated, slowly growing masses, and thus are often quite large before coming to clinical attention.

Anatomic location is the most important prognostic factor, because ALT/WDL does not metastasize unless dedifferentiation has occurred (see later discussion). Indeed, although the terms *ALT* and *WDL* are synonymous, tumors in surgically resectable locations in the limbs and trunk are labeled *ALT* because wide excision is curative. In contrast, those located in the retroperitoneum and mediastinum, where wide excision is difficult, are referred to as *WDL*, because repeated and uncontrolled local recurrences are very common, and mortality is high even in the absence of dedifferentiation.

Histologically, ALT/WDL is divided into four subtypes (without prognostic implications): adipocytic (lipoma-like), sclerosing, inflammatory, and spindle cell, of which the first two are the most common.[28] In general, ALT/WDL is composed of relatively mature adipose tissue with significant variation in cell size and varying degrees of nuclear atypia in adipocytes and stromal cells, particularly within fibrous septa. Lipoblasts are often present; however, they are frequently rare and are not required for the diagnosis. Sclerosing ALT/WDL is characterized by collagenous stroma containing pleomorphic hyperchromatic stromal cells and is most common in the retroperitoneum and paratesticular region. Inflammatory ALT/WDL is rare but is important to recognize in that it can be misdiagnosed as Hodgkin's lymphoma, as other sarcomas, or even as a nonneoplastic process, because of the very extensive chronic inflammation present. Spindle-cell ALT/WDL is composed of only mildly atypical spindle cells admixed with adipocytes and lipoblasts (in many cases), in a fibrous or myxoid stroma.

Dedifferentiation, defined by progression to nonlipogenic, often morphologically high-grade sarcoma, occurs in approximately 10% of ALT/WDL. It most commonly occurs in the retroperitoneum, because dedifferentiation appears to be a time-dependent (or size-dependent) phenomenon, and retroperitoneal ALT/WDL often remains asymptomatic until it reaches a large size and tends to have a protracted clinical course because of its relative unresectability. Histologically, dedifferentiated liposarcoma (DDLPS) is characterized in most cases by an abrupt transition from ALT/WDL areas to variably pleomorphic spindle cell sarcomatous areas. DDLPS has a 40% to 50% local recurrence rate, a 15% metastatic rate, and a 30% 5-year mortality rate.[29]

Genetics and Molecular Pathogenesis

Giant marker and supernumerary ring chromosomes are the hallmark of ALT/WDL and are also present in DDLPS.[30] These giant ring and marker chromosomes contain massive amplification of the 12q13-15 chromosomal region.[31] A number of other chromosomal regions, including 12q21-22 and 1q21-25, have also been shown to be co-amplified. The *p53* regulator *MDM2*, located in 12q14-15, is consistently amplified in ALT/WDL, typically in association with neighboring genes, including *CDK4*, *SAS*, and *HMGA2*. Expression of both MDM2 and CDK4 has been shown to be a sensitive and specific marker to distinguish ALT/WDL from benign lipoma (which can be a challenge, especially if there is coexistent fat necrosis).[32] *MDM2* negatively regulates the tumor suppressor *p53* by targeting it for ubiquitin-mediated destruction.[33] Approximately 30% to 40% of sarcomas in general display *MDM2* overamplification, and 38% of tumors in mice overexpressing *MDM2* are sarcomas.[34] Nutlins, a class of small-molecule inhibitors of *MDM2*,[35] have been shown to have growth inhibitory effects on ALT/WDL cell lines, raising their consideration as a new potential therapeutic option.[36]

Although not fully elucidated, recent data have begun to unravel the molecular pathogenesis of tumor progression from ALT/WDL to DDLPS. DDLPS differ cytogenetically from ALT/WDL in that they often show additional complex karyotypic changes, including involvement of 1p32 and 6q23.[37] DDLPS also have been shown to possess a greater degree of 12q amplification than ALT/WDL. Chibon and colleagues[38] identified a role for amplification of *ASK1 (MAP3K5)* a mitogen-activated protein *(MAP)* kinase kinase on 6q23 that is upstream of JNK kinase and *JUN*, in dedifferentiation via inhibition of adipocyte differentiation. More recently, a role for *JUN* oncogene amplification on 1p32 in dedifferentiation has been shown.[39,40] Overexpression of *JUN* results in downregulation of genes involved in adipocytic differentiation, possibly via its direct interaction with CCAAT-enhancer-binding protein beta *(C/EBPβ)*, a transcription factor involved in adipogenesis.[39] Interestingly, *JUN* amplification has been found in the WDL component (separate from the DDLPS component) of a number of DDLS, suggesting that JUN amplification occurs before dedifferentiation in at least a subset of WDL.[37,40] Inhibition of JUN expression in DDLPS with amplified *JUN* results in a reduction in proliferation and tumor growth, both in vitro and in vivo, suggesting a key role for *JUN* upregulation in DDLPS.[40] These in vivo data, however, suggest that *JUN* amplification is likely not to be sufficient to promote dedifferentiation, as inhibition of JUN expression does not promote adipocyte differentiation in DDLPS tumors.[40]

Despite its histology, DDLPS has a less aggressive clinical course than most high-grade pleomorphic sarcomas in adults. The latter tumors typically display both *MDM2* and *p53* alterations that correlate with a poor prognosis.[41] In contrast, although there are conflicting data regarding the frequency of *p53* mutation in DDLPS,[42-44] it is likely mutated in only a minority of these tumors.

Gastrointestinal Stromal Tumor

Clinical Description and Pathology

Gastrointestinal stromal tumors (GISTs) are the most common sarcomas of the gastrointestinal tract.[45] The overall age range at presentation is very broad; however, the majority of tumors are diagnosed in patients older than 50. GISTs occur with equal incidence in both males and females and can occur at any location in the gastrointestinal tract. The most common site is the stomach (50%), followed by the small intestine (25%), large intestine (10%), esophagus (5%), and, rarely, the gallbladder, appendix, or pancreas. GISTs can also arise at sites outside the tubular gastrointestinal tract, including the retroperitoneum, pelvis, mesentery, and omentum, although these extragastrointestinal GISTs account for only about 10% of all GISTs. Clinical presentation may include anemia secondary to gastrointestinal bleeding, early satiety, and intestinal obstruction; however, smaller GISTs are also quite often identified as incidental findings.

The most common metastatic sites for GISTs are intra-abdominal, namely the liver, peritoneum, omentum, and mesentery. GISTs rarely spread to lymph nodes (other than in the pediatric subtype) or to extra-abdominal sites, and when they do, it tends to be late in the course of disease. The most important predictors of metastasis are tumor size and mitotic index, with a size less than 5 cm and a mitotic index less than 5 per 50 high power fields (hpf) conferring a low risk of aggressive behavior and a size greater than 10 cm and/or a mitotic index greater than 10 per 50 hpf conferring a high risk of aggressive behavior.[46] Very occasionally, however, even small GISTs (less than 2 cm) with a low proliferative rate can behave aggressively; thus follow-up is recommended even for lesions with a low relative risk of metastasis.

Based on cytomorphology, GISTs can be divided histologically into three categories: spindle cell, epithelioid, and mixed epithelioid and spindle-cell type. Epithelioid areas are composed of cells growing in sheets or nests, with round nuclei and fairly abundant eosinophilic cytoplasm (Figure 44-2, *A*). The spindled cells are typically monomorphic, with vesicular chromatin and palely eosinophilic, almost syncytial cytoplasm, growing in short fascicles (see Figure 44-2, *B*). Pleomorphism is rare. Additional characteristic features of both spindle-cell and epithelioid GISTs are the presence of

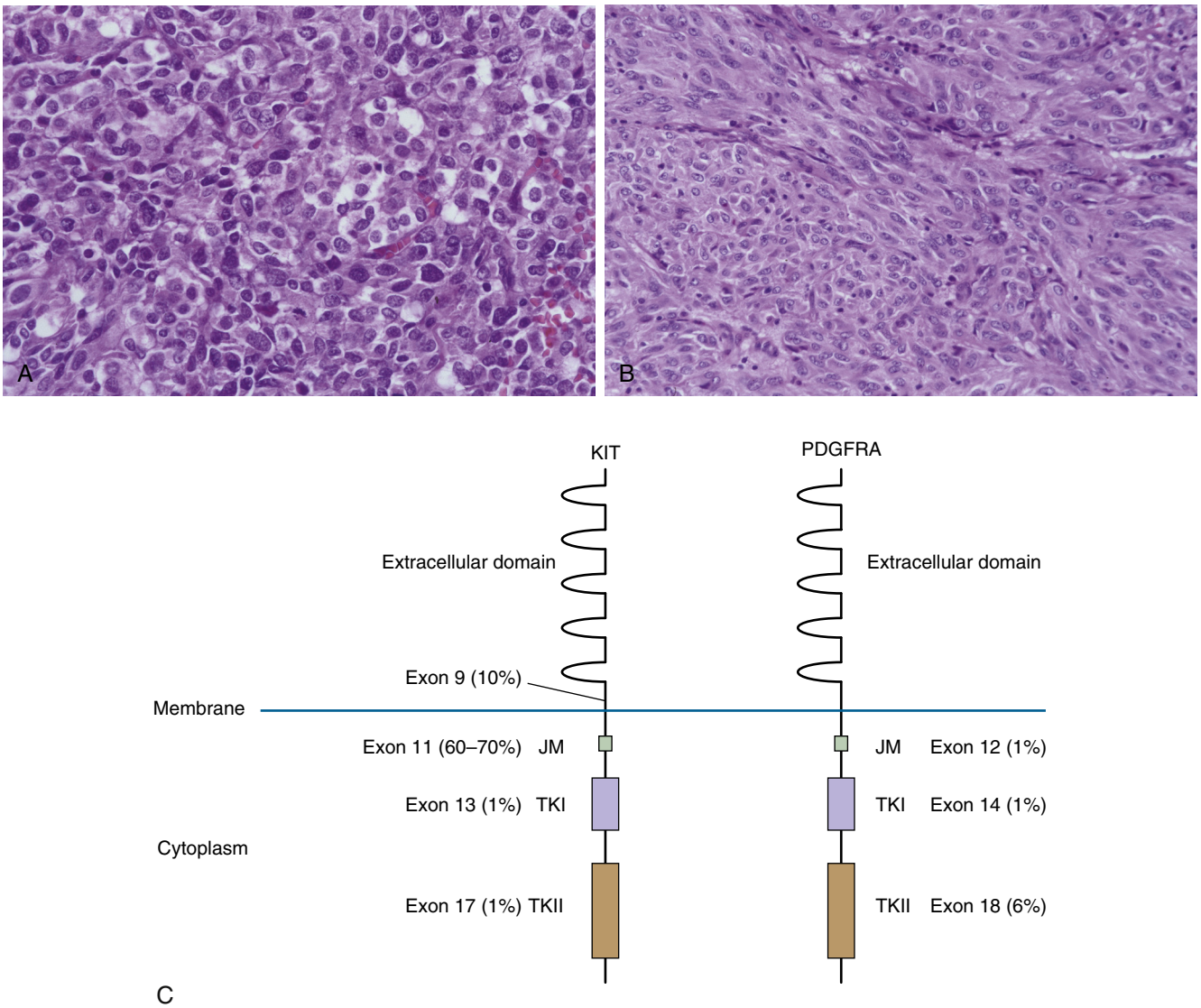


Figure 44-2 Gastrointestinal stromal tumor (GIST) **(A)** Epithelioid GIST, showing cells with round nuclei and fairly abundant eosinophilic cytoplasm, perinuclear vacuoles, and fibrillary eosinophilic cytoplasm. **(B)** Spindle-cell GIST, demonstrating the typical monomorphic cells with vesicular chromatin and palely eosinophilic syncytial-appearing cytoplasm, growing in short fascicles. **(C)** Diagram of c-Kit and PDGFRA, demonstrating localization and frequency of mutations occurring in sporadic GIST. Approximately 5%-10% of GIST lack either c-Kit or PDGFRA mutations. *JM,* Juxtamembrane domain; *TKI,* tyrosine kinase domain I; *TKII,* tyrosine kinase domain II; *TM,* transmembrane domain.

perinuclear vacuoles (especially in gastric lesions) and the fibrillary nature of the cytoplasm.

GISTs frequently stain positively for smooth muscle markers (at least 30% to 40% are positive for smooth muscle actin or caldesmon). In the past, this finding, combined with the eosinophilic quality of the cytoplasm, caused these tumors to often be mistaken for smooth muscle tumors. GISTs were also not uncommonly mistaken for neural tumors because they can exhibit prominent nuclear palisading, mimicking a nerve sheath tumor, and can occasionally (approximately 5%) stain positively for *S100* protein, a marker common to neural tumors. However, unlike other mesenchymal tumors of the gastrointestinal tract, the vast majority (95%) of GISTs are positive for the tyrosine kinase receptor *c-kit* (KIT) (also known as *CD117*), typically in a diffuse cytoplasmic, dotlike, or membranous pattern.[45] This KIT immunopositivity reflects the effect of activating mutations in KIT (see later discussion) and has greatly improved the reproducibility of GIST diagnosis. More recently, antibodies to DOG-1, a protein highly expressed in GISTs, have been shown to be highly specific, and more sensitive than KIT, in the diagnosis of GIST.[47,48]

In the gastrointestinal tract, KIT is also expressed in interstitial cells of Cajal (ICC), gut pacemaker cells exhibiting both smooth muscle and neuronal differentiation ultrastructurally and immunohistochemically, which are important for intestinal peristalsis.[49] Given the similarities between ICC and GISTs both ultrastructurally and

immunohistochemically, it is believed that most GISTs show differentiation toward ICC.

Genetics and Molecular Pathogenesis

Approximately 85% to 90% of GISTs harbor activating *KIT* or *PDGFRA* mutations[45] (including in familial GIST syndrome, in which patients have germline activating mutations in *KIT* or *PDGFRA*, which are inherited in an autosomal dominant fashion).[50] In addition to GISTs, patients with familial GIST syndrome also have ICC hyperplasia, and those with specific mutations in exon 11 of *KIT* have abnormal skin pigmentation and mastocytosis. The proto-oncogene KIT is a type III receptor tyrosine kinase related to platelet-derived growth factor receptor (PDGFR) and is required for melanogenesis, myelopoiesis, fertility, and ICC and mast-cell development.[51,52] It is a transmembrane protein whose ligand is stem-cell factor (SCF). Binding of SCF to KIT results in KIT activation via autophosphorylation, leading to downstream phosphorylation of key signal transduction proteins and regulation of a number of cell processes, including proliferation, survival, cell adhesion, and differentiation.[53] In GISTs, *KIT* mutations cluster mainly in four exons: exon 9 (extracellular transmembrane domain), exon 11 (intracellular juxtamembrane domain, exon 13 (initial portion of the kinase domain), and exon 17 (kinase activation loop) (see Figure 44-2, C). Current data suggest that exon 11 mutations are the most frequent (60% to 70% of GISTs).[45] Mutations in exons 13 and 17 are rare.

Platelet-derived growth factor receptor A *(PDGFRA)* is mutated in approximately 5% of GISTs, resulting in constitutive activation and downstream activation of signal transduction molecules similar to those affected by KIT activating mutations.[54] *KIT* and *PDGFRA* mutations are mutually exclusive and the mutations found in *PDGFRA* map to similar domains on the protein to those found in *KIT* (see Figure 44-2, C). GISTs with *PDGFRA* mutations tend to exhibit epithelioid rather than spindle-cell morphology and tend to be more common in the stomach.

A mouse knockin model expressing constitutively active KIT has demonstrated that this constitutive activation is necessary and sufficient for GIST tumorigenesis;[55,56] thus mutation in either KIT or *PDGFRA* is likely an early step in GIST pathogenesis. However, other recurrent genetic changes have been shown to occur. These include loss of chromosomes 14q, followed by loss of 22, 11p, 9p, or 1p.[45] Loss of 9p, on which the tumor suppressor *p16(Ink4a)* is located, appears to be associated with a worse prognosis. Gains of chromosome 5p, 20q, 17q, or 8q are also associated with a more aggressive clinical behavior; however, correlation of tumor progression with specific genes in those regions has yet to be demonstrated.

Approximately 5% to 10% of GISTs lack either *KIT* or *PDGFRA* mutations (wild-type [WT] GIST). These *KIT*/*PDGFR*-intact GISTs tend to occur in the pediatric population, as well as in the setting of specific genetic syndromes, namely, neurofibromatosis type I/Von Recklinghausen's neurofibromatosis (NFI); Carney triad (CT); and Carney-Stratakis syndrome (CSS). CT, for which a clear genetic inheritance pattern or germline mutation has not been found to date,[57] consists of extra-adrenal paragangliomas, pulmonary chondromas, and multifocal epithelioid GISTs of the stomach.[58] The CSS is characterized by paragangliomas and GISTs and is due to germline mutations in succinate dehydrogenase subunits B, C, or D *(SDHB*, *SDHC*, *SDHD)*, inherited in an autosomal dominant fashion.[59,60] *SDH* is a mitochondrial enzyme complex that functions in the Krebs cycle. Recent data have also identified *SDHB* or *SDHC* germline mutations in patients with WT GISTs without a personal or family history to suggest CT or CSS,[61] as well as somatic mutation of *SDHA*.[62] In addition, loss of *SDHB* protein expression and decreased *SDH* complex activity have been demonstrated in WT GISTs in patients without germline or tumoral *SDH* mutation, further supporting a role for the *SDH* complex in tumorigenesis in these genetically distinct GISTs.[61] Although the majority of *SDHB*-deficient GISTs occurs in young patients (hence being designated *pediatric-type*), comparable lesions with distinctive clinicopathologic features occur more rarely in adults.[63,64]

GISTs are not responsive to conventional chemotherapy or radiation, and thus, before the identification of *KIT* mutations in GISTs and the development of targeted therapy, the outlook for patients with clinically aggressive GISTs was grim. Imatinib mesylate, also known as Gleevec, binds to and inhibits the ATP-binding pocket of both KIT and PDGFRA and is approved to treat patients with metastatic and/or unresectable GISTs. Mutational status appears to be important in predicting response to imatinib, in that patients with exon 11 *KIT* mutations have a significantly better response than patients with exon 9 *KIT* mutations or without *KIT* or *PDGFRA* mutations.[65] Interestingly, GISTs with either exon 9 *KIT* mutations or without *KIT* or *PDGFRA* mutations are more likely to be responsive to sunitinib, a second-line tyrosine kinase inhibitor approved as therapy for imatinib-resistant GISTs.[66] Given the role of *SDH* mutation and/or deficiency in a significant subset of WT GISTs, protein expression of *SDHB* has been proposed as a means to initially triage GISTs into those that likely harbor *KIT* or *PDGFRA* mutations (type 1; intact *SDHB* expression), and those with likely *SDH* complex deficiency (type 2; absent *SDHB* expression[67,68]). This subclassification has important clinical implications, as the type 2

GISTs (WT GISTs) usually do not respond to imatinib. It remains important, however, to consider *SDHB* expression alone with caution, because of potential difficulties in staining interpretation, and because some tumors classified as type 1 via *SDHB* positivity (namely, GISTs occurring in the setting of NF1) do not contain *KIT* or *PDGFRA* mutations and do not typically respond to imatinib.[69]

Unfortunately, a significant number of patients eventually develop secondary resistance to imatinib as well as to sunitinib, primarily via secondary mutations in the KIT kinase domain or through KIT amplification.[66,70,71] KIT has been shown to activate a number of key signal transduction pathways, including the mitogen-activated protein kinase *(MAPK)*, Janus kinase/signal transducers and activators of transcription *(JAK/STAT)*, Src family of tyrosine kinases *(SFK)*, Ras/extracellular regulated kinase *(Ras/Raf/Erk)*, and *PI3* kinase signaling pathways.[53,72] Of these pathways, in vitro and in vivo data demonstrate that the *PI3* kinase, *MAPK*, and *SFK* pathways are activated in GISTs.[72,73] In contrast, phosphorylation of the *JAK/STAT* kinases *STAT1* and *STAT3* was not dependent on oncogenic KIT signaling, and *STAT5* phosphorylation was not detected in either primary GISTs or GIST cell lines. In addition, inhibition of the JAK/STAT pathway did not inhibit proliferation in GIST cell lines, arguing against a significant role for this pathway in GIST oncogenesis.[74] Elucidation of the comparable importance of downstream targets of KIT in GIST tumorigenesis has important therapeutic implications, particularly for the development of treatment strategies in imatinib-resistant tumors. For example, a secondary mutation in BRAF kinase (a kinase mutated in several cancers, particularly melanoma, papillary thyroid carcinoma, and a subset of colorectal cancers) has been identified in an imatinib-resistant GIST, suggesting a possible role for the potent BRAF inhibitor vemurafenib as second-line therapy.[75] Interestingly, *BRAF* somatic mutation has also been identified as a rare event in WT GIST.[75]

Conclusion

The identification of specific chromosomal aberrations in a significant subset of sarcomas has improved our ability both to diagnose and to develop novel treatment strategies for these tumors. Just as the elucidation of specific translocations in another category of mesodermally derived neoplasms—leukemias—allowed the development of directed chemotherapeutics (e.g., chronic myelogenous leukemia and imatinib), so, too, the discovery of specific recurrent genetic mutations in sarcomas provides opportunities for intelligent design of targeted therapies. This is in contrast to the two thirds of sarcomas that lack recurrent genetic abnormalities and which often possess complex karyotypes, which unfortunately include most pleomorphic/spindle-cell sarcomas of older adults. Application of comprehensive genomic analysis over the past several years to these and other sarcomas, however, has provided new insight into novel oncogenic drivers of these tumors, as well as an enhancement in our ability to predict clinical behavior.[76,77] This improved understanding of sarcoma genetics is providing new potential therapeutic targets as well as diagnostic and prognostic biomarkers.[2,78]

Key References

1. Antonescu CR. The role of genetic testing in soft tissue sarcoma. *Histopathology*. 2006;48(1):13-21.
2. Taylor BS, et al. Advances in sarcoma genomics and new therapeutic targets. *Nat Rev Cancer*. 2011;11(8):541-557. *
3. Perot G, et al. Constant p53 pathway inactivation in a large series of soft tissue sarcomas with complex genetics. *Am J Pathol*. 2010;177(4):2080-2090.
4. de Bruijn DR, Nap JP, van Kessel AG. The (epi)genetics of human synovial sarcoma. *Genes Chromosomes Cancer*. 2007;46(2):107-117.
10. Haldar M, et al. A conditional mouse model of synovial sarcoma: insights into a myogenic origin. *Cancer Cell*. 2007;11(4):375-388. *
12. Guillou L, et al. Histologic grade, but not SYT-SSX fusion type, is an important prognostic factor in patients with synovial sarcoma: a multicenter, retrospective analysis. *J Clin Oncol*. 2004;22(20):4040-4050.
15. Oliveira AM, Fletcher CD. Molecular prognostication for soft tissue sarcomas: are we ready yet? *J Clin Oncol*. 2004;22(20):4031-4034.
16. Perani M, et al. Conserved SNH domain of the proto-oncoprotein SYT interacts with components of the human chromatin remodelling complexes, while the QPGY repeat domain forms homo-oligomers. *Oncogene*. 2003;22(50):8156-8167.
21. Su L, et al. Deconstruction of the SS18-SSX Fusion Oncoprotein Complex: Insights into Disease Etiology and Therapeutics. *Cancer Cell*. 2012;21(3):333-347. *
22. Foo WC, et al. Immunohistochemical staining for TLE1 distinguishes synovial sarcoma from histologic mimics. *Am J Clin Pathol*. 2011;135(6):839-844.
23. Jagdis A, et al. Prospective evaluation of TLE1 as a diagnostic immunohistochemical marker in synovial sarcoma. *Am J Surg Pathol*. 2009;33(12):1743-1751.
24. Knosel T, et al. TLE1 is a robust diagnostic biomarker for synovial sarcomas and correlates with t(X;18): analysis of 319 cases. *Eur J Cancer*. 2010;46(6):1170-1176.
25. Lubieniecka JM, et al. Histone deacetylase inhibitors reverse SS18-SSX-mediated polycomb silencing of the tumor suppressor early growth response 1 in synovial sarcoma. *Cancer Res*. 2008;68(11):4303-4310.
26. Ito T, et al. Significant growth suppression of synovial sarcomas by the histone deacetylase inhibitor FK228 in vitro and in vivo. *Cancer Lett*. 2005;224(2):311-319.
27. Friedrichs N, et al. Phosphatidylinositol-3'-kinase/AKT signaling is essential in synovial sarcoma. *Int J Cancer*. 2011;129(7):1564-1575.
30. Arrigoni G, Doglioni C. Atypical lipomatous tumor: molecular characterization. *Curr Opin Oncol*. 2004;16(4):355-358.
31. Dal Cin P, et al. Cytogenetic and fluorescence in situ hybridization investigation of ring chromosomes characterizing a specific pathologic subgroup of adipose tissue tumors. *Cancer Genet Cytogenet*. 1993;68(2):85-90.
32. Binh MB, et al. MDM2 and CDK4 immunostainings are useful adjuncts in diagnosing well-differentiated and dedifferentiated liposarcoma subtypes: a comparative analysis of 559 soft tissue neoplasms with genetic data. *Am J Surg Pathol*. 2005;29(10):1340-1347.

33. Moll UM, Petrenko O. The MDM2-p53 interaction. *Mol Cancer Res*. 2003;1(14):1001-1008.
35. Vassilev LT, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*. 2004;303(5659):844-848.
36. Singer S, et al. Gene expression profiling of liposarcoma identifies distinct biological types/subtypes and potential therapeutic targets in well-differentiated and dedifferentiated liposarcoma. *Cancer Res*. 2007;67(14):6626-6636. *
38. Chibon F, et al. ASK1 (MAP3K5) as a potential therapeutic target in malignant fibrous histiocytomas with 12q14-q15 and 6q23 amplifications. *Genes Chromosomes Cancer*. 2004;40(1):32-37.
39. Mariani O, et al. JUN oncogene amplification and overexpression block adipocytic differentiation in highly aggressive sarcomas. *Cancer Cell*. 2007;11(4):361-374. *
40. Snyder EL, et al. c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipocytic differentiation programme. *J Pathol*. 2009;218(3):292-300.
45. Rubin BP. Gastrointestinal stromal tumours: an update. *Histopathology*. 2006;48(1):83-96.
46. Miettinen M, Lasota J. Gastrointestinal stromal tumors: pathology and prognosis at different sites. *Semin Diagn Pathol*. 2006;23(2):70-83.
47. Espinosa I, et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. *Am J Surg Pathol*. 2008;32(2):210-218.
48. Liegl B, et al. Monoclonal antibody DOG1.1 shows higher sensitivity than KIT in the diagnosis of gastrointestinal stromal tumors, including unusual subtypes. *Am J Surg Pathol*. 2009;33(3):437-446.
53. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. *Cell Mol Life Sci*. 2004;61(19-20):2535-2548.
54. Corless CL, et al. PDGFRA mutations in gastrointestinal stromal tumors: frequency, spectrum and in vitro sensitivity to imatinib. *J Clin Oncol*. 2005;23(23):5357-5364.
55. Sommer G, et al. Gastrointestinal stromal tumors in a mouse model by targeted mutation of the Kit receptor tyrosine kinase. *Proc Natl Acad Sci U S A*. 2003;100(11):6706-6711.
56. Rubin BP, et al. A knock-in mouse model of gastrointestinal stromal tumor harboring kit K641E. *Cancer Res*. 2005;65(15):6631-6639.
57. Stratakis CA, Carney JA. The triad of paragangliomas, gastric stromal tumours and pulmonary chondromas (Carney triad), and the dyad of paragangliomas and gastric stromal sarcomas (Carney-Stratakis syndrome): molecular genetics and clinical implications. *J Intern Med*. 2009;266(1):43-52.
59. Pasini B, et al. Clinical and molecular genetics of patients with the Carney-Stratakis syndrome and germline mutations of the genes coding for the succinate dehydrogenase subunits SDHB, SDHC, and SDHD. *Eur J Hum Genet*. 2008;16(1):79-88.
60. McWhinney SR, et al. Familial gastrointestinal stromal tumors and germ-line mutations. *N Engl J Med*. 2007;357(10):1054-1056. *
61. Janeway KA, et al. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc Natl Acad Sci U S A*. 2011;108(1):314-318.
62. Pantaleo MA, et al. SDHA loss-of-function mutations in KIT-PDGFRA wild-type gastrointestinal stromal tumors identified by massively parallel sequencing. *J Natl Cancer Inst*. 2011;103(12):983-987.
63. Miettinen M, et al. Succinate dehydrogenase-deficient GISTs: a clinicopathologic, immunohistochemical, and molecular genetic study of 66 gastric GISTs with predilection to young age. *Am J Surg Pathol*. 2011;35(11):1712-1721.
64. Rege TA, et al. "Pediatric-type" gastrointestinal stromal tumors in adults: distinctive histology predicts genotype and clinical behavior. *Am J Surg Pathol*. 2011;35(4):495-504.
65. Heinrich MC, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 2003;21(23):4342-4349.
66. Heinrich MC, et al. Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. *J Clin Oncol*. 2008;26(33):5352-5359.
67. Gill AJ, et al. Immunohistochemistry for SDHB divides gastrointestinal stromal tumors (GISTs) into 2 distinct types. *Am J Surg Pathol*. 2010;34(5):636-644.
68. Gill AJ, et al. "Pediatric-type" gastrointestinal stromal tumors are SDHB negative ("type 2") GISTs. *Am J Surg Pathol*. 2011;35(8):1245-1247; author reply 1247-1248.
69. Wang JH, Lasota J, Miettinen M. Succinate Dehydrogenase Subunit B (SDHB) Is Expressed in Neurofibromatosis 1-Associated Gastrointestinal Stromal Tumors (Gists): Implications for the SDHB Expression Based Classification of Gists. *J Cancer*. 2011;2:90-93.
70. Antonescu CR, et al. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. *Clin Cancer Res*. 2005;11(11):4182-4190.
71. Liegl B, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. *J Pathol*. 2008;216(1):64-74.
72. Rossi F, et al. Imatinib upregulates compensatory integrin signaling in a mouse model of gastrointestinal stromal tumor and is more effective when combined with dasatinib. *Mol Cancer Res*. 2010;8(9):1271-1283.
73. Duensing A, et al. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene*. 2004;23(22):3999-4006.
74. Bauer S, et al. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. *Oncogene*. 2007;26(54):7560-7568. *
75. Agaram NP, et al. Novel V600E BRAF mutations in imatinib-naive and imatinib-resistant gastrointestinal stromal tumors. *Genes Chromosomes Cancer*. 2008;47(10):853-859.
76. Chibon F, et al. Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. *Nat Med*. 2010;16(7):781-787. *
77. Gibault L, et al. New insights in sarcoma oncogenesis: a comprehensive analysis of a large series of 160 soft tissue sarcomas with complex genomics. *J Pathol*. 2011;223(1):64-71.
78. Barretina J, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet*. 2010;42(8):715-721. *

This page intentionally left blank

References

1. Antonescu CR. The role of genetic testing in soft tissue sarcoma. *Histopathology*. 2006;48(1):13-21.
2. Taylor BS, et al. Advances in sarcoma genomics and new therapeutic targets. *Nat Rev Cancer*. 2011;11(8):541-557. *
3. Perot G, et al. Constant p53 pathway inactivation in a large series of soft tissue sarcomas with complex genetics. *Am J Pathol*. 2010;177(4):2080-2090.
4. de Bruijn DR, Nap JP, van Kessel AG. The (epi)genetics of human synovial sarcoma. *Genes Chromosomes Cancer*. 2007;46(2):107-117.
5. Cadman NL, Soule EH, Kelly PJ. Synovial Sarcoma; an Analysis of 34 Tumors. *Cancer*. 1965;18:613-627.
6. Morton MJ, et al. MR imaging of synovial sarcoma. *AJR Am J Roentgenol*. 1991;156(2):337-340.
7. Lewis JJ, et al. Synovial sarcoma: a multivariate analysis of prognostic factors in 112 patients with primary localized tumors of the extremity. *J Clin Oncol*. 2000;18(10):2087-2094.
8. Bergh P, et al. Synovial sarcoma: identification of low and high risk groups. *Cancer*. 1999;85(12):2596-2607.
9. Skytting B. Synovial sarcoma. A Scandinavian Sarcoma Group project. *Acta Orthop Scand Suppl*. 2000;291:1-28.
10. Haldar M, et al. A conditional mouse model of synovial sarcoma: insights into a myogenic origin. *Cancer Cell*. 2007;11(4):375-388. *
11. dos Santos NR, de Bruijn DR, van Kessel AG. Molecular mechanisms underlying human synovial sarcoma development. *Genes Chromosomes Cancer*. 2001;30(1):1-14.
12. Guillou L, et al. Histologic grade, but not SYT-SSX fusion type, is an important prognostic factor in patients with synovial sarcoma: a multicenter, retrospective analysis. *J Clin Oncol*. 2004;22(20):4040-4050.
13. Ladanyi M, et al. Impact of SYT-SSX fusion type on the clinical behavior of synovial sarcoma: a multi-institutional retrospective study of 243 patients. *Cancer Res*. 2002;62(1):135-140.
14. Kawai A, et al. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. *N Engl J Med*. 1998;338(3):153-160.
15. Oliveira AM, Fletcher CD. Molecular prognostication for soft tissue sarcomas: are we ready yet? *J Clin Oncol*. 2004;22(20):4031-4034.
16. Perani M, et al. Conserved SNH domain of the proto-oncoprotein SYT interacts with components of the human chromatin remodelling complexes, while the QPGY repeat domain forms homo-oligomers. *Oncogene*. 2003;22(50):8156-8167.
17. Eid JE, et al. p300 interacts with the nuclear proto-oncoprotein SYT as part of the active control of cell adhesion. *Cell*. 2000;102(6):839-848.
18. Lim FL, et al. A KRAB-related domain and a novel transcription repression domain in proteins encoded by SSX genes that are disrupted in human sarcomas. *Oncogene*. 1998;17(15):2013-2018.
19. dos Santos NR, et al. Delineation of the protein domains responsible for SYT, SSX, and SYT-SSX nuclear localization. *Exp Cell Res*. 2000;256(1):192-202.
20. Nagai M, et al. Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. *Proc Natl Acad Sci U S A*. 2001;98(7):3843-3848.
21. Su L, et al. Deconstruction of the SS18-SSX Fusion Oncoprotein Complex: Insights into Disease Etiology and Therapeutics. *Cancer Cell*. 2012;21(3):333-347. *
22. Foo WC, et al. Immunohistochemical staining for TLE1 distinguishes synovial sarcoma from histologic mimics. *Am J Clin Pathol*. 2011;135(6):839-844.
23. Jagdis A, et al. Prospective evaluation of TLE1 as a diagnostic immunohistochemical marker in synovial sarcoma. *Am J Surg Pathol*. 2009;33(12):1743-1751.
24. Knosel T, et al. TLE1 is a robust diagnostic biomarker for synovial sarcomas and correlates with t(X;18): analysis of 319 cases. *Eur J Cancer*. 2010;46(6):1170-1176.
25. Lubieniecka JM, et al. Histone deacetylase inhibitors reverse SS18-SSX-mediated polycomb silencing of the tumor suppressor early growth response 1 in synovial sarcoma. *Cancer Res*. 2008;68(11):4303-4310.
26. Ito T, et al. Significant growth suppression of synovial sarcomas by the histone deacetylase inhibitor FK228 in vitro and in vivo. *Cancer Lett*. 2005;224(2):311-319.
27. Friedrichs N, et al. Phosphatidylinositol-3'-kinase/AKT signaling is essential in synovial sarcoma. *Int J Cancer*. 2011;129(7):1564-1575.
28. Laurino L, et al. Well-differentiated liposarcoma (atypical lipomatous tumors). *Semin Diagn Pathol*. 2001;18(4):258-262.
29. Nascimento AG. Dedifferentiated liposarcoma. *Semin Diagn Pathol*. 2001;18(4):263-266.
30. Arrigoni G, Doglioni C. Atypical lipomatous tumor: molecular characterization. *Curr Opin Oncol*. 2004;16(4):355-358.
31. Dal Cin P, et al. Cytogenetic and fluorescence in situ hybridization investigation of ring chromosomes characterizing a specific pathologic subgroup of adipose tissue tumors. *Cancer Genet Cytogenet*. 1993;68(2):85-90.
32. Binh MB, et al. MDM2 and CDK4 immunostainings are useful adjuncts in diagnosing well-differentiated and dedifferentiated liposarcoma subtypes: a comparative analysis of 559 soft tissue neoplasms with genetic data. *Am J Surg Pathol*. 2005;29(10):1340-1347.
33. Moll UM, Petrenko O. The MDM2-p53 interaction. *Mol Cancer Res*. 2003;1(14):1001-1008.
34. Jones SN, et al. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. *Proc Natl Acad Sci U S A*. 1998;95(26):15608-15612.
35. Vassilev LT, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*. 2004;303(5659):844-848.
36. Singer S, et al. Gene expression profiling of liposarcoma identifies distinct biological types/subtypes and potential therapeutic targets in well-differentiated and dedifferentiated liposarcoma. *Cancer Res*. 2007;67(14):6626-6636. *
37. Chibon F, et al. A subgroup of malignant fibrous histiocytomas is associated with genetic changes similar to those of well-differentiated liposarcomas. *Cancer Genet Cytogenet*. 2002;139(1):24-29.
38. Chibon F, et al. ASK1 (MAP3K5) as a potential therapeutic target in malignant fibrous histiocytomas with 12q14-q15 and 6q23 amplifications. *Genes Chromosomes Cancer*. 2004;40(1):32-37.
39. Mariani O, et al. JUN oncogene amplification and overexpression block adipocytic differentiation in highly aggressive sarcomas. *Cancer Cell*. 2007;11(4):361-374. *
40. Snyder EL, et al. c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipocytic differentiation programme. *J Pathol*. 2009;218(3):292-300.
41. Cordon-Cardo C, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res*. 1994;54(3):794-799.
42. Dei Tos AP, et al. Molecular abnormalities of the p53 pathway in dedifferentiated liposarcoma. *J Pathol*. 1997;181(1):8-13.
43. Pilotti S, et al. Distinct mdm2/p53 expression patterns in liposarcoma subgroups: implications for different pathogenetic mechanisms. *J Pathol*. 1997;181(1):14-24.
44. Schneider-Stock R, et al. MDM2 amplification and loss of heterozygosity at Rb and p53 genes: no simultaneous alterations in the oncogenesis of liposarcomas. *J Cancer Res Clin Oncol*. 1998;124(10):532-540.
45. Rubin BP. Gastrointestinal stromal tumours: an update. *Histopathology*. 2006;48(1):83-96.
46. Miettinen M, Lasota J. Gastrointestinal stromal tumors: pathology and prognosis at different sites. *Semin Diagn Pathol*. 2006;23(2):70-83.
47. Espinosa I, et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. *Am J Surg Pathol*. 2008;32(2):210-218.
48. Liegl B, et al. Monoclonal antibody DOG1.1 shows higher sensitivity than KIT in the diagnosis of gastrointestinal stromal tumors, including unusual subtypes. *Am J Surg Pathol*. 2009;33(3):437-446.
49. Kindblom LG, et al. Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. *Am J Pathol*. 1998;152(5):1259-1269.
50. Nishida T, et al. Familial gastrointestinal stromal tumours with germline mutation of the KIT gene. *Nat Genet*. 1998;19(4):323-324.
51. Ashman LK. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol*. 1999;31(10):1037-1051.

52. Huizinga JD, et al. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature*. 1995;373(6512):347-349.
53. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. *Cell Mol Life Sci*. 2004;61(19-20):2535-2548.
54. Corless CL, et al. PDGFRA mutations in gastrointestinal stromal tumors: frequency, spectrum and in vitro sensitivity to imatinib. *J Clin Oncol*. 2005;23(23):5357-5364.
55. Sommer G, et al. Gastrointestinal stromal tumors in a mouse model by targeted mutation of the Kit receptor tyrosine kinase. *Proc Natl Acad Sci U S A*. 2003;100(11):6706-6711.
56. Rubin BP, et al. A knock-in mouse model of gastrointestinal stromal tumor harboring kit K641E. *Cancer Res*. 2005;65(15):6631-6639.
57. Stratakis CA, Carney JA. The triad of paragangliomas, gastric stromal tumours and pulmonary chondromas (Carney triad), and the dyad of paragangliomas and gastric stromal sarcomas (Carney-Stratakis syndrome): molecular genetics and clinical implications. *J Intern Med*. 2009;266(1):43-52.
58. Carney JA, et al. The triad of gastric leiomyosarcoma, functioning extra-adrenal paraganglioma and pulmonary chondroma. *N Engl J Med*. 1977;296(26):1517-1518.
59. Pasini B, et al. Clinical and molecular genetics of patients with the Carney-Stratakis syndrome and germline mutations of the genes coding for the succinate dehydrogenase subunits SDHB, SDHC, and SDHD. *Eur J Hum Genet*. 2008;16(1):79-88.
60. McWhinney SR, et al. Familial gastrointestinal stromal tumors and germ-line mutations. *N Engl J Med*. 2007;357(10):1054-1056. *
61. Janeway KA, et al. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc Natl Acad Sci U S A*. 2011;108(1):314-318.
62. Pantaleo MA, et al. SDHA loss-of-function mutations in KIT-PDGFRA wild-type gastrointestinal stromal tumors identified by massively parallel sequencing. *J Natl Cancer Inst*. 2011;103(12):983-987.
63. Miettinen M, et al. Succinate dehydrogenase-deficient GISTs: a clinicopathologic, immunohistochemical, and molecular genetic study of 66 gastric GISTs with predilection to young age. *Am J Surg Pathol*. 2011;35(11):1712-1721.
64. Rege TA, et al. "Pediatric-type" gastrointestinal stromal tumors in adults: distinctive histology predicts genotype and clinical behavior. *Am J Surg Pathol*. 2011;35(4):495-504.
65. Heinrich MC, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol*. 2003;21(23):4342-4349.
66. Heinrich MC, et al. Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. *J Clin Oncol*. 2008;26(33):5352-5359.
67. Gill AJ, et al. Immunohistochemistry for SDHB divides gastrointestinal stromal tumors (GISTs) into 2 distinct types. *Am J Surg Pathol*. 2010;34(5):636-644.
68. Gill AJ, et al. "Pediatric-type" gastrointestinal stromal tumors are SDHB negative ("type 2") GISTs. *Am J Surg Pathol*. 2011;35(8):1245-1247; author reply 1247-1248.
69. Wang JH, Lasota J, Miettinen M. Succinate Dehydrogenase Subunit B (SDHB) Is Expressed in Neurofibromatosis 1-Associated Gastrointestinal Stromal Tumors (Gists): Implications for the SDHB Expression Based Classification of Gists. *J Cancer*. 2011;2:90-93.
70. Antonescu CR, et al. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. *Clin Cancer Res*. 2005;11(11):4182-4190.
71. Liegl B, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. *J Pathol*. 2008;216(1):64-74.
72. Rossi F, et al. Imatinib upregulates compensatory integrin signaling in a mouse model of gastrointestinal stromal tumor and is more effective when combined with dasatinib. *Mol Cancer Res*. 2010;8(9):1271-1283.
73. Duensing A, et al. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). *Oncogene*. 2004;23(22):3999-4006.
74. Bauer S, et al. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. *Oncogene*. 2007;26(54):7560-7568. *
75. Agaram NP, et al. Novel V600E BRAF mutations in imatinib-naive and imatinib-resistant gastrointestinal stromal tumors. *Genes Chromosomes Cancer*. 2008;47(10):853-859.
76. Chibon F, et al. Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. *Nat Med*. 2010;16(7):781-787. *
77. Gibault L, et al. New insights in sarcoma oncogenesis: a comprehensive analysis of a large series of 160 soft tissue sarcomas with complex genomics. *J Pathol*. 2011;223(1):64-71.
78. Barretina J, et al. Subtype-specific genomic alterations define new targets for soft-tissue sarcoma therapy. *Nat Genet*. 2010;42(8):715-721. *

John Mendelsohn

45 From Bench to Bedside with Targeted Therapies

Targeted therapy of human disease celebrated its 100th birthday in 2011. A century ago Paul Ehrlich, a German pathologist, produced the first "rationally designed" drug, which selectively targeted the microorganism *Treponema pallidum*, the cause of syphilis. The project was the outcome of a thoughtful hypothesis based on the observation that certain organic dyes were selectively taken up by some cells and infectious agents, and not by others. Ehrlich postulated the presence of specific receptors on cells and bacteria to which the dyes bound and were internalized. He then proposed to attach a toxic molecule (in this case, arsenic) to a dye molecule that was selective for a receptor on the bacterium, with the goal of producing selective cytotoxicity against the bacterium[1] (Figure 45-1).

To carry out this work in an efficient and systematic way, he invented an array-like approach to his research. A series of large organic molecules were attached to arsenic by his colleague, Sahachiro Hata, a synthetic chemist, and then were tested by Ehrlich for selective toxicity. Number 606—actually, a modification of the original number 606—was effective and became arsphenamine (Salvarsan). To demonstrate efficacy and lack of toxicity, Ehrlich first performed experiments with rabbit models of syphilis (mice resist the infection), followed by what we would call Phase I and II clinical trials with patients—keeping careful records. Finally, after obtaining positive results, he scaled up production and marketed the first synthetic "blockbuster" chemotherapeutic agent in 1911, a mere decade after initiating the project. It remained the standard of care until antibiotics were discovered.

The subsequent production of numerous chemical agents that can cure disease or prolong life is a magnificent story of moving discoveries from the research bench to the patient's bedside, and from the patient back to the research bench. In the history of chemotherapy for cancer, progress developed in two phases. During the first phase, from 1946 to the mid-1980s, the drugs produced were screened for efficacy primarily by assaying cytotoxicity against cancer cells in culture and in animal models, and then in patients. Most of the drugs acted by interfering with DNA replication or cell division. It is remarkable that until the1970s, medical students were taught that the biochemical and molecular abnormalities in cancer cells were quantitative (altered amounts) rather than qualitative (altered chemical composition and structure). During the second phase, which began in the mid-1980s, the targets for drug development became products of the aberrantly functioning genes that produce cancer. This progression was made possible by new knowledge about oncogenes and suppressor genes, and by new technologies for accurately detecting abnormalities in DNA and proteins.

Drug Development

The First Phase

Chemotherapy for cancer began in the 1940s, with the development of agents that target and eliminate malignant cells. The observed toxicity of mustard gas against blood leukocytes led Louis Goodman and Alfred Gillman to produce a form of this DNA alkylating agent, which could be delivered intravenously.[2] This chemotherapy, named nitrogen mustard, produced objective clinical responses in patients with lymphoma. Another novel therapy that targeted DNA synthesis was developed by Sidney Farber. Knowing that folic acid was required for DNA synthesis, he worked with a synthetic chemist to produce folate antagonists. Amethopterin and later Aminopterin (methotrexate) produced dramatic but short-lived responses in children with acute lymphoblastic leukemia.[3] With these two reports, the pharmacological therapy of cancer was born.

Although the title of this chapter is "From Bench to Bedside," it is important to emphasize that the movement of

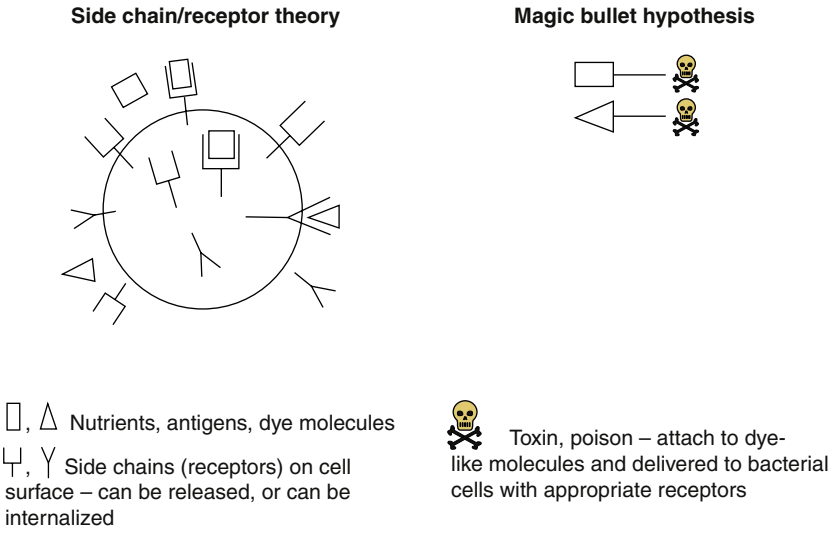


Figure 45-1 Paul Ehrlich: birth of targeted therapy, 1911.

ideas is invariably in both directions, back and forth between clinical researchers and the laboratory researchers. Nitrogen mustard was developed because physicians observed destruction of leukocytes and lymphocytes in soldiers gassed during World War I. The antifolate, Aminopterin, was developed because Sidney Farber tried treating leukemic children with folic acid (reasoning that their malignant cells appeared similar to cells observed with folate deficiency), and the acuteness of their disease became worse, not better. These observations, in turn, stimulated and informed laboratory research.

For the next 40 years, great progress was made in developing additional chemotherapeutic anticancer agents. Two approaches were used. The first approach involved synthesis of specific targeted agents—as with methotrexate. The next success was 6-mercaptopurine, another inhibitor of DNA synthesis, produced by George Hitchings and Gertrude Elion and shown by Joseph Burchenal to be effective in acute leukemia.[4] The synthesis of new therapeutic molecules has been greatly enhanced by the development of three-dimensional models of target molecules, enabling chemists to design small lead molecules and test them using in silico computer-based screening.

The second approach involved screening of large numbers of natural products and led to the discovery of taxanes and camptothecin. These successes were followed by discovery of the anticancer properties of vinca alkaloids, platinum-based agents, nitrosoureas, and anthracyclines, all of which remain in use today.

The pharmaceutical industry has used these two general approaches—synthesis of new compounds and broad screening of natural products—to develop many therapies against cancer and bring them to the clinic for investigation in therapeutic clinical trials[5] (Figure 45-2). The successful chemotherapeutic agents that have been developed are summarized in Chapter 46. Today new methods of targeting and screening are being used to find therapies that counteract the function, or loss of function, of the products of aberrant genes that cause cancer (see later discussion).

Two other major breakthroughs in the development of cancer therapies during this first phase deserve emphasis. The first is the development of combinations of therapies administered simultaneously. This was applied to leukemia by Emil Freireich, Emil Frei, and James Holland in 1956, soon after combinations of antibiotics were found to produce enhanced efficacy against bacterial infections such as tuberculosis. The principle is to use two or more agents that provide additive killing capacities against a target, but with different toxic side effects that are not additive. In the late 1950s and early 1960s, this principle was first applied successfully in a series of clinical trials by these investigators and others for the treatment of childhood leukemia,[6] and it was first used for the successful treatment of a solid tumor, testicular cancer, by M. C. Li and colleagues.[7]

Leukemia researchers also pioneered the idea that treatment must continue beyond the time that the cancer is clinically detectable, in order to prevent recurrence due to the persistence of subclinical disease. Today this is standard practice for the care of many types of cancer. As with the combination therapy studies, the results of leukemia research in murine models provided the rationale for these studies.

A second major breakthrough in cancer therapy and the treatment of many diseases took advantage of a new technology invented by Kohler and Milstein, which enabled production of large quantities of a monoclonal antibody raised against a specific antigen.[8] This technique was rapidly applied to the production of antibodies targeting molecules on the surface of cancer cells. Major clinical responses in lymphoma patients were reported by Ronald Levy and colleagues in 1982, with an anti-idiotype antibody against the specific immunoglobulin molecule expressed on the surface

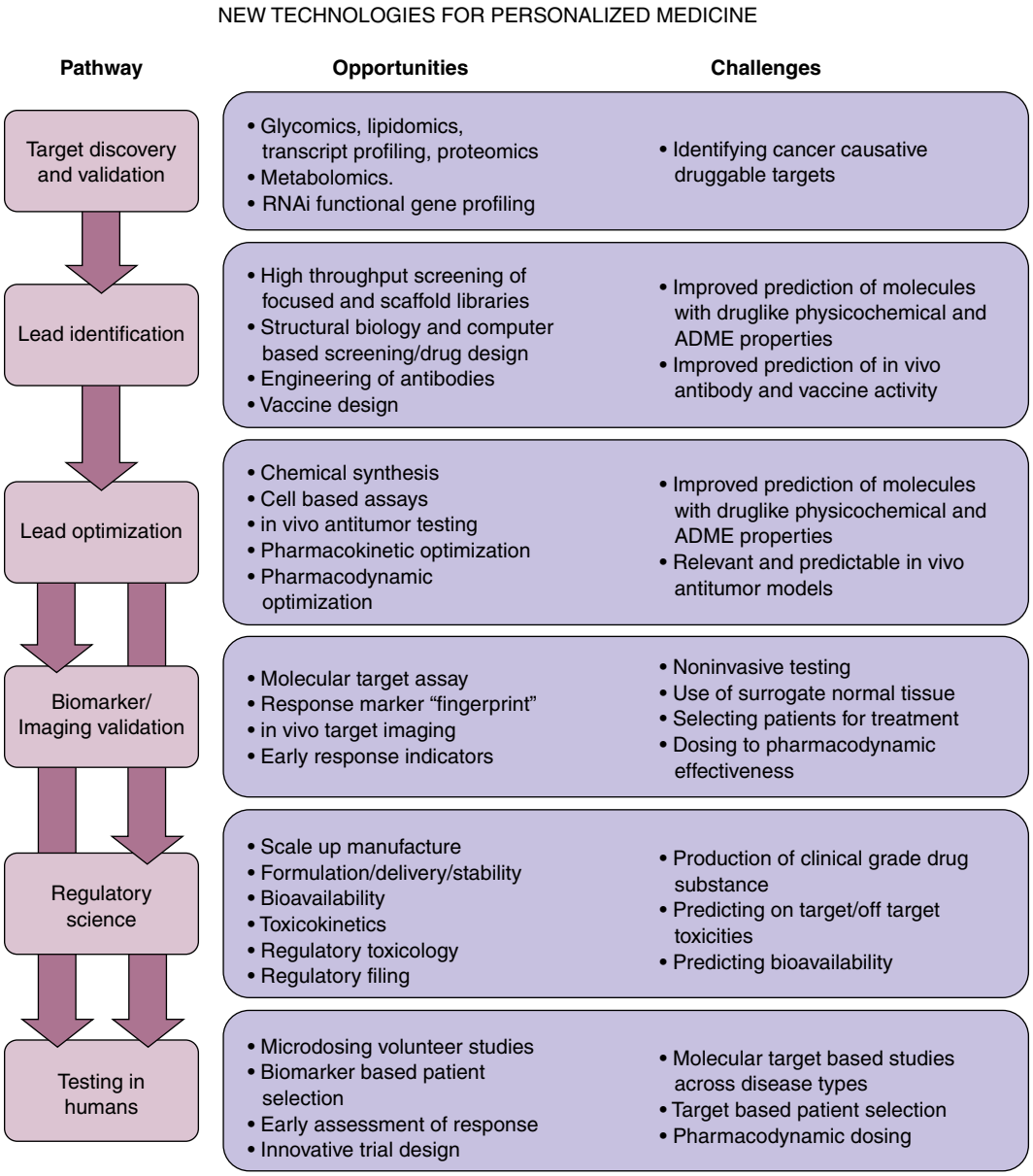


Figure 45-2 New approaches and challenges for personalized cancer medicine Therapy development is shown as linear process, whereas in reality it is iterative in all its stages.

of the patients' malignant B cells.[9] Today, nearly half of the agents that are approved by the U.S. Food and Drug Administration (FDA) for the treatment of cancer are monoclonal antibodies, a revolution in targeted cancer therapy that has occurred in the past three decades.

The Second Phase

Why not produce an anticancer therapy that targets a molecule that is known to cause cancer? A preliminary to this approach dates back to the 1940s and 1950s, when Charles Huggins discovered that depriving prostate cancer patients of androgen and breast cancer patients of estrogen can result in remission of their disease.[10] Both approaches are used in the clinic to this day, but the method has evolved from surgical removal of organs producing these hormones to targeted drugs that act on hormone receptors or hormone production.

The target for therapy switched from cytoplasmic hormone receptors to growth factor receptors on the cell surface in the early 1980s. Our group first hypothesized that inhibition of a receptor function might inhibit tumor cell growth (Table 45-1). We produced a monoclonal antibody, cetuximab, which binds to the EGF receptor, blocks activation of the receptor's tyrosine kinase by its ligands, EGF and TGFα, and inhibits proliferation of cancer cells in culture and in human tumor xenografts.[11-13] This was followed by reports on trastuzumab, a monoclonal antibody that binds to the closely related HER-2 receptor.[14] It was during this period that the term *oncogene* was first used, and these were the first

Table 45-1 Rationale for Targeting EGF Receptors, 1980

- EGF characterized 1962. EGF receptor characterized 1975-80. (Cohen)
- Autocrine hypothesis 1980. (Todaro and Sporn)
- Tyrosine kinase first identified in three molecules: src oncogene, EGF receptor and PDGF receptor (Hunter, Erickson, Cohen).
- EGF receptor commonly overexpression in cancers (Ozanne, others)
- Preferential addiction of cancer cells to essential nutrients and growth factors
- Receptor inhibition by circulating autoantibodies can produce stable physiologic change (disease) in humans: myasthenia gravis, hyperthyroidism, insulin resistance

experimental cancer therapies that targeted the products of an oncogene—in these cases a receptor tyrosine kinase. Since the mid-1980s, many oral chemotherapeutic agents have been developed that target these two tyrosine kinases and many other kinases, located both in receptors and free in the cytoplasm.

Trastuzumab received regulatory approval for the treatment of breast cancer expressing high levels of HER-2 in 1998.[15] This was followed by imatinib against the product of the BCR-ABL gene translocation, another tyrosine kinase, in patients with chronic myelocytic leukemia in 2001.[16] Cetuximab entered clinical trials in 1990 and was approved for colorectal cancer in 2004[17,18] and for head and neck cancer in 2006. These approaches to experimental therapeutics created a new paradigm for the development of therapies against cancer.

Three breakthroughs in knowledge and technology converged to greatly accelerate this change in therapeutic research on cancer:

(1) We have learned that accumulated genetic aberrations are the cause of cancer. This dates from observations by Bishop and Varmus in experiments exploring viral carcinogenesis with the SRC oncogene, published in 1976, showing that the genetic abnormality was intrinsic to human cells, not the Rous sarcoma virus.[19] Today, hundreds of genes are known to be altered in human cancers.

(2) There are more than 800 new drugs under development at pharmaceutical and biotechnology companies and universities, which are designed to target the products of the aberrantly functioning genes that cause cancer.

(3) New instruments and analytic techniques have reduced sequencing of a human genome from a 10-year, $3 billion project to a 10-day, $5000 project, which allows us to interrogate an individual patient's cancer to detect genetic aberrations in the patient's tumor. The speed and cost continue to improve dramatically.

As a result of this confluence of discoveries, there is pressure from all stakeholders in cancer care for development of new approaches to the treatment of cancer—using the genomic and molecular analysis of an individual patient's cancer to select the targeted therapy that is most likely to provide clinical benefit. Although earlier approaches provided many successful chemotherapeutic agents and continue to be important, the new targeted approach to developing new cancer therapies dominates the field today. These new therapies are designed to target a particular gene product that has been shown to contribute to the malignant phenotype of cancer cells in correlative studies of molecular pathological data and clinical data and in laboratory experiments with cultured cells and animal models.[20,21]

There have been some spectacular successes in clinical trials with experimental targeted therapies that enrolled only patients whose cancer was known to harbor an aberrancy in the target of that experimental drug or antibody. These successes have resulted in substantial prolongation of patients' lives for many months or years.

The first example of this approach to reach the clinic involved trastuzumab, the monoclonal antibody against the HER-2 receptor. It is overexpressed on 20% to 25% of breast cancers and was found to be a biomarker predicting a worse prognosis in these patients. Clinical activity of trastuzumab was demonstrated in early studies only among patients whose cancers expressed high levels of HER-2. Genentech adopted a new paradigm for designing a randomized Phase III clinical trial, investigating standard chemotherapy with or without trastuzumab for treatment of patients with advanced, metastatic breast cancer. Only patients with high expression of HER-2 in their tumors were eligible to enroll. This enriched the trial with patients more likely to respond, and converted what would otherwise have been a negative study (too few responders) into a positive study, leading to FDA approval in 2002.[15] The optimal clinical situations for the use of trastuzumab have been refined continuously, as a result of dozens of clinical trials over the ensuing decade (see Chapters 36 and 50).

A second and most dramatic example involves the development of the ABL tyrosine kinase inhibitor imatinib for the treatment of patients with chronic myelocytic leukemia (CML), whose leukocytes nearly always carry a BCR/ABL gene rearrangement. Before this treatment, the median survival of patients with this disease was 3½ years. Today most patients on this and follow-up drugs live in remission more than 5 years, and some may reach a normal lifespan.[16] The incidence of CML is only 4000 cases per year. A drug against this disease was not considered to be a potential "blockbuster" compared with a treatment for lung cancer (over 200,000 cases/year). Movement from the research laboratory in a pharmaceutical company to clinical trials was encouraged by the persistent efforts of an academic clinician scientist, Brian Druker, who performed preclinical studies in his laboratory demonstrating efficacy against CML cells and

then led the first clinical trials. The premise, which turned out to be prescient, was that blocking the abnormally active ABL tyrosine kinase—a product of the BCR-ABL rearrangement in patients with early CML—might produce especially effective responses, because this was the only known genetic abnormality in these patients. In contrast, most cancers have many genetic aberrations that contribute to their malignant activities at the time they are first detected.

A third example involves oral inhibitors of the EGF receptor tyrosine kinase, gefitinib and erlotinib (TKIs). In a series of very large and expensive randomized trials of chemotherapy plus or minus one or the other of these TKIs in patients with advanced lung cancer, the results showed no significant overall benefit from adding the TKI. However, a few patients had a substantial prolongation of survival. When it was discovered that mutations were present in the gene encoding the EGF receptor in the lung cancers of many of the patients who responded well,[22-24] clinical trials were repeated with the requirement of an EGF receptor mutation for enrollment. The positive response rates for advanced lung cancer patients with a mutated EGF receptor were confirmed.[25] This time, in the selected patient population, the results were positive in the majority of patients. Thus, a "failed" new targeted therapy was converted into a successful treatment for the 10% of lung cancer patients with mutated EGF receptor genes in their tumors, and the median duration of survival for those patients has doubled. In fact, for these patients the TKI therapy was more effective than chemotherapy, whereas for patients without the mutation, chemotherapy gave better outcomes than treatment with a TKI.[25] This "resurrection of a cancer drug" brought clinical benefits to selected patients and economic benefits to the pharmaceutical companies, which were now able to market their new targeted drugs. This was a wake-up call to the industry, strongly suggesting the value of a new, selective approach to clinical trials with new targeted cancer treatments.

Among the lessons from these examples is the observation that for each case the circumstances were different, and an understanding of both the molecular biology and the clinical disease was critical in the development of a novel therapy that targeted a molecular abnormality in these patients' cancers.

Von Hoff was the first to report on a clinical trial in which patient enrollment was governed by the result of a molecular analysis of tumors, using immunohistochemistry, fluorescence in situ hybridization, and, primarily, gene expression arrays. Of 66 patients treated with commercially available drugs that were felt to match the detected molecular markers, 27% had a progression-free survival at least 30% longer compared with the response to the previous drug(s) they had received.[26] In this study, the patients served as their own controls, and gene mutations were not analyzed.

Recent examples have demonstrated the utility of identifying the targeted genetic aberration in the tumors of patients who enroll in clinical trials with experimental targeted therapies at the earliest stages.

The Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) trial for patients with advanced, heavily treated non–small-cell lung cancer was the first reported study with a panel of experimental drugs that (1) required a fresh tumor biopsy for genetic and molecular assays to detect particular aberrations that could serve as biomarkers for assigning therapy and (2) introduced an adaptive randomization design to assign a patient to receive one of the four experimental therapies that did, or did not, target an abnormality detected in that patient's cancer. Essentially, this was a panel of four separate Phase II trials, testing the efficacy of "rationally" assigning patients to a particular trial. Importantly, nearly 100% of patients who were offered participation agreed to the biopsy procedure as a condition for receiving access to one of the experimental targeted therapies in a randomized study. The endpoint of the trial was improved duration of progression-free survival, which was subsequently confirmed by improved overall survival. The results showed that there were four situations where the results of the biomarker assays predicted a significant improvement in response, or in one case, a worse response, to an experimental drug targeting that biomarker.[27] This suggested that preselection of patients for a panel of Phase II studies of new therapeutic agents based on biomarkers showing abnormalities in the targets of these agents is useful and may improve clinical outcomes.

Another trial at MD Anderson Cancer Center retrospectively analyzed response rates and survival of patients treated with experimental drugs that matched genetic abnormalities in their cancers. Over a 4-year period, more than 1000 patients with advanced, heavily treated cancers were tested for mutations in hot spots in 11 genes and for loss of expression of PTEN. An experimental drug was often available targeting the detected genetic aberration for most of their genes in ongoing Phase I/II trials. A total of 291 patients with genetic aberrations could be evaluated for response to an experimental targeted therapy. Of these, 175 received a targeted therapy that matched an aberrant gene target in their cancer, and 116 were treated with nonmatched therapy (based on nonavailability of matched therapy at the time). The overall response rate of the first group was 27% (including two complete responses), significantly greater than the second group, which was 5%. Median overall survival was 13.4 months compared to 9.0 months, a 49% increase.[28] In most reports analyzing the results of Phase I trials, the main objective is determination of maximal tolerated dose and

toxicities, and the objective response rate to new therapies is less than 10%. This trial, which achieved a much higher response rate, was a nonrandomized study and involved many types of cancer and many separate clinical trials of new drugs at varying doses. Although the results support the benefit of matching experimental therapy to genetic aberrations, this conclusion now must be confirmed in a randomized study. Today a number of cancer centers are carrying out prospective clinical trials using next-generation sequencing technology to assess cancers and using the results for assigning patients to trials of experimental targeted therapies.

Two recent highly successful Phase II trials of experimental targeted drugs took an approach to testing a new experimental therapy similar to that used in the registration trial of trastuzumab, the trials of TKIs in patients with mutated EGF receptors, and in most of the patients with CML treated with imatinib. Each trial studied a single drug. Large numbers of patients were screened for enrollment, based on the presence of the genetic aberration that the experimental drug was designed to target.

In the first clinical trial, crizotinib, which already was in trials targeting the EML4-ALK rearrangement in lymphoma, was tested for activity against patients with this rearrangement in their lung cancer. This was found to occur in less than 5% of lung cancers. More than 1700 patients were screened at multiple institutions to populate a Phase II trial with 82 preselected patients. The drug was successful, with a response rate of 57%, an additional 35% achieving stable disease, and a median time to progression of greater than 6 months at the time of publication.[29] These results led to successful follow-up trials and FDA approval, within only 5 years of discovering this genetic aberration in small numbers of lung cancer patients (the drug was already available).

Another clinical trial explored the efficacy of vemurafenib which was designed to block the activity of BRAF, a gene that was reported to be mutated in many patients with melanoma. The complete plus partial response rate with advanced melanoma patients whose cancer had the targeted BRAF mutation was over 70%, with a median duration of 7 months.[30] FDA approval followed soon afterward.

These were remarkable results in patients with advanced cancers that were well known to be minimally responsive or resistant to chemotherapy. It is clear that genomic screening can improve response rates with targeted therapies, benefiting patients and shortening the time required for testing an experimental therapy. However, we will not know how generalizable this conclusion is until the results of all such targeted trials—positive and negative—are reported and shared. Negative trials of experimental drugs are not typically reported, although they inform decision making by the pharmaceutical company that is developing them. Meanwhile, the results just described support pursuing this approach to testing targeted experimental therapies in a screened subset of cancer patients.

Next Steps in Drug Development

Progress will be hastened if a number of challenges are addressed—some involving gaps in scientific knowledge and technology, and others involving changes in the clinical trials process.

Scientific Knowledge and Technology

A major scientific challenge is the need to balance the tremendous power of next-generation genomic sequencing and other technologies for interrogating molecular aberrations in a patient's cancer with the mandate to keep down the costs of clinical care. The most sophisticated genomic tests (see Chapter 24) add substantial costs for instrumentation and data analysis, although they may reduce the cost of care in the long run. An added factor is the requirement in the United States that all tests performed to guide clinical care must be carried out in laboratories with Clinical Laboratory Improvement Amendment (CLIA) certification, which ensures high standards, but adds costs. Similar standards are used in Europe. Finally, sophisticated, deep-level next-generation sequencing often identifies hundreds of genetic aberrations in human cancer specimens,[31] and most of these are not actionable because their significance in the tumor's biology and the patient's disease is not known, or drugs that target them are not yet available.

For these reasons, clinical testing for genomic aberrations in most medical centers currently is limited to assays that measure mutations (insertions, deletions, or substitutions of nucleotides) in selected genes, alterations in gene copy number, and structural rearrangements in selected genes[32] (Figure 45-3). Available experimental or approved drugs that target the products of aberrant genes are currently limited to a few dozen genes. At the author's institution, we currently are sequencing 740 hot spots in 46 genes. This detects only the first of the three categories of genomic analysis listed above. To capture all three categories and expand the gene coverage, we are planning to move to the targeted deep sequencing of the exons (open reading frames) of a few hundred genes. Both of these approaches to sequencing enable multiplexed assays on many genes to be run on a single extracted sample of tumor DNA. This is a huge advance over the technology just a few years ago, when selected regions of genes were sequenced in a separate assay for each individual mutation, and the amount of tumor

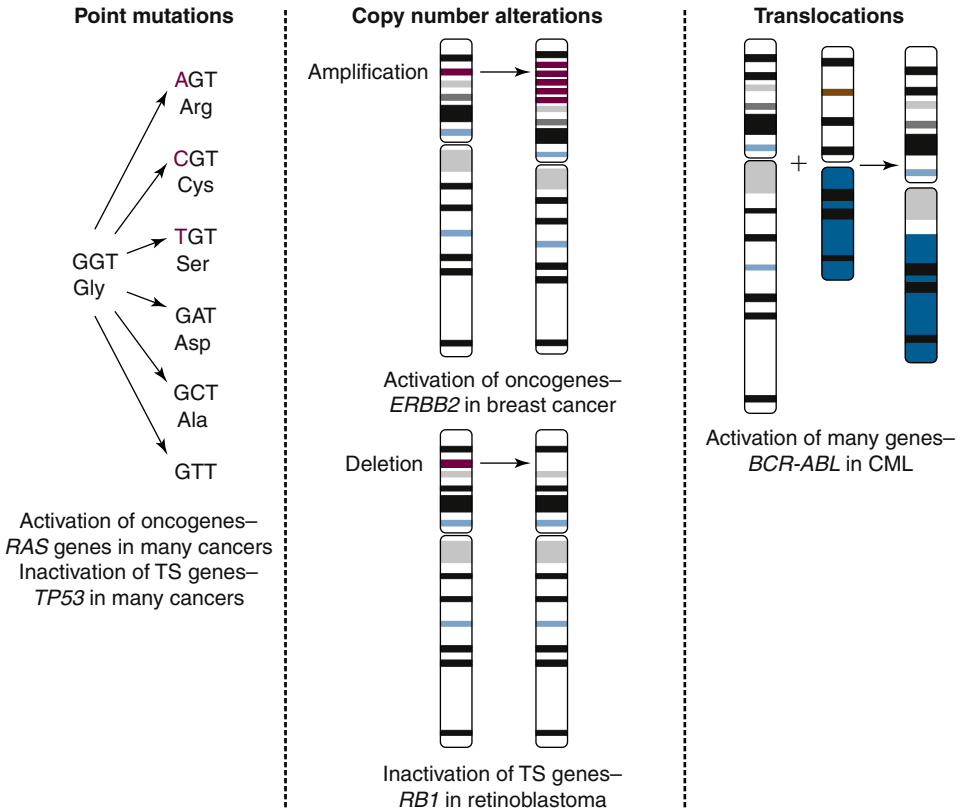


Figure 45-3 The major classes of genomic alterations that give rise to cancer. *CML*, Chronic myelogenous leukemia; *TS*, tumor suppressor.[32]

tissue available rapidly became a limiting constraint. A number of cancer centers have started programs assaying DNA in patient's cancers to guide therapy (e.g., Refs. 33, 34).

With 800 experimental targeted drugs in the clinical pipeline and a few dozen already approved, current second-generation sequencing of advanced solid tumors yields a match between a genetic aberration and a targeted drug about 40% of the time. The yield is higher in reported cases of deep sequencing with more complete genomic analysis. The ingenuity of companies developing new technologies for detecting genetic aberrations guarantees that advancements will continue to enable more rapid and complete analysis for less cost. The limiting factors have become the need for bioinformatics and computational tools and for trained personnel to use these tools to interpret the sequencing data.

Two key rate-limiting gaps in scientific knowledge were identified recently in a report from the President's Council of Advisors on Science and Technology.[35] These were (1) identifying and validating the protein targets that are critical to the survival of cancer cells and are "druggable," and (2) predicting more effectively the efficacy and toxicity of candidate drugs before high investments of funds and time are made in clinical trials.

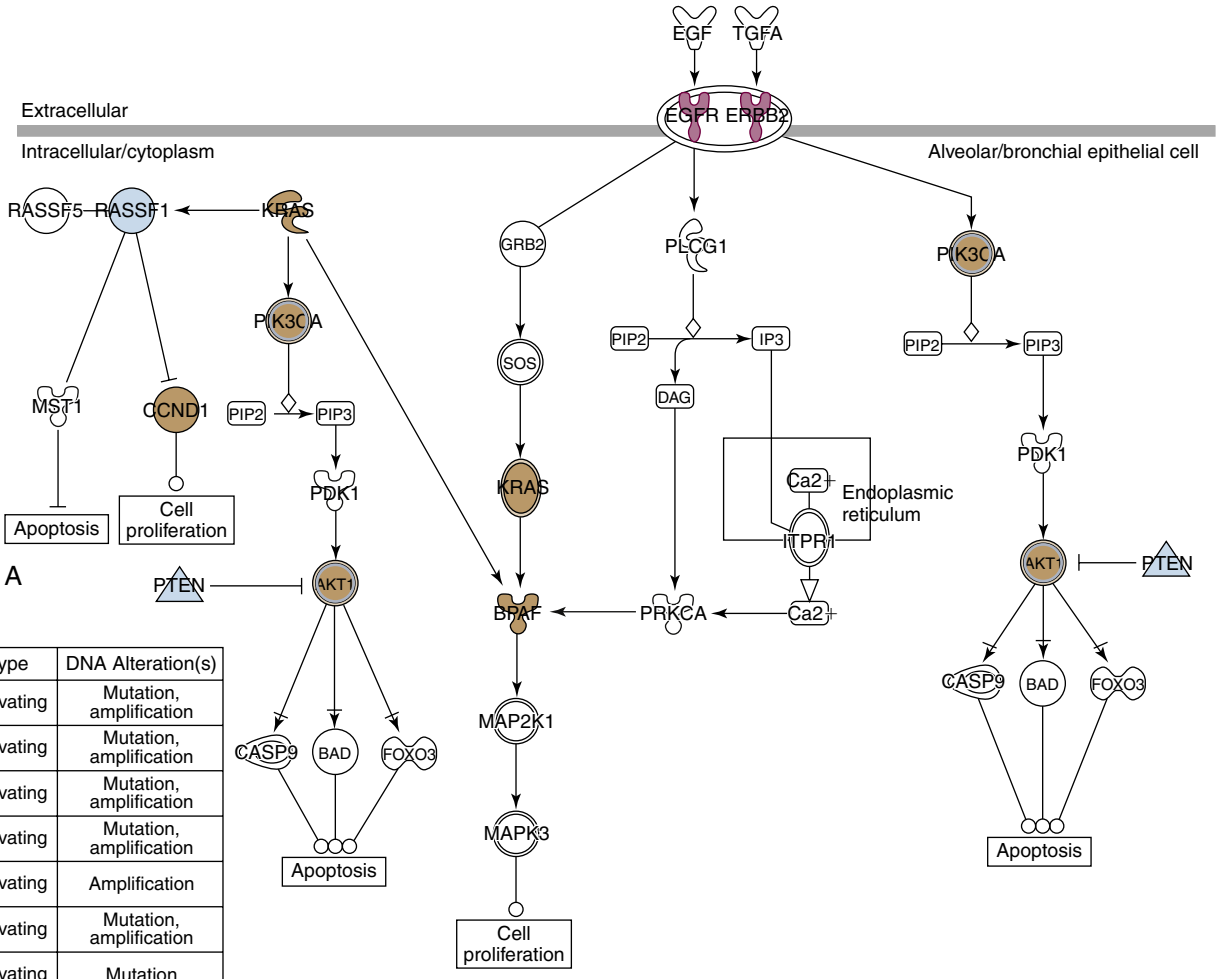
The new fields of systems biology and computational biology have emerged to address the first of these questions. Through extensive computer-based analysis of data from biological studies on the biochemical steps that activate or suppress molecular processes in cells, investigators have been able to draw complex maps of interconnected pathways that regulate the activities of proteins in those cells. Candidate "drivers" that form pivotal controlling nodes in these interconnected pathways are identified in a number of ways: (1) They may connect multiple signaling pathways; (2) they may be recurrently mutated in human cancer specimens; (3) introduction of specific RNAi's can block a signaling pathway that changes major phenotypic functions in the cell (such as maintaining viability); and (4) knockin or knockout of the gene's activity in genetically engineered mouse models (GEMMs) results in molecular alterations, changing the phenotype in significant ways that affect malignant behavior.[36,37] It is important to note that up to now, most targeted therapies discussed in this chapter were developed with earlier approaches that involved identification of abnormal functioning of a target in biological experiments with cancer cells, not because of detection of aberrant genes in broad screens of human cancers.

The Human Cancer Genome Atlas and additional data collected from genomic testing of cancers in multiple centers will identify more genetic aberrations that are cancer drivers and potential targets for new drugs. These new approaches will also enable identification of driver genes that are present at low frequencies—for example, less than 5%—in few types or only one type of cancer. It must be cautioned that a mutation in a known oncogene does not necessarily mean that it

is a driver in a particular cancer, and a gene could have "driver" status in a cancer without a genetic aberration, working instead through amplification or an epigenetic mechanism, or through changes in expression of message or posttranslational modification of the protein it encodes. It also must be emphasized that a driver molecule may be activated by a variety of mechanisms related to its situation in one or more activated regulatory pathways[38] (Figure 45-4).

The ultimate driver gene is one which is so critical to a cancer cell's survival that when its function is blocked, the cell dies, typically by apoptosis. The observation of this phenomenon led to the "addiction hypothesis" of Weinstein, who postulated that in cancer cells that are driven by a mutation in a critical regulatory pathway, alternative pathways that could promote or bypass the driving gene's activity may be downregulated to the point where, unlike the situation in normal cells, they may be irretrievably damaged when the driving, addicting gene is suddenly blocked by a targeted therapy.[39]

The identification of a driver gene does not invariably lead to creation of a drug effective against the gene's product. The tyrosine kinase oncogenes have turned out to be "druggable" targets, and a few dozen new agents against these targets are in clinical use or in clinical trials—both antibodies against receptors on the cell surface and their ligands, and low



Gene	Type	DNA Alteration(s)
EGFR	Activating	Mutation, amplification
ERBB2	Activating	Mutation, amplification
KRAS	Activating	Mutation, amplification
PIK3CA	Activating	Mutation, amplification
CCND1	Activating	Amplification
AKT1	Activating	Mutation, amplification
BRAF	Activating	Mutation
PTEN	Inactivating	Mutation, deletion methylation
RASSF-1	Inactivating	Mutation, deletion methylation

B

Figure 45-4 Activation of EGFR signaling in non–small-cell lung cancer (NSCLC) can occur via disruption of several different components at multiple levels of the pathway. (A) Different proteins in the EGFR pathway can be activated (*red*) or inactivated (*blue*) by underlying genetic or epigenetic changes at the DNA level, leading to aberrant pathway activity and oncogenic signaling in NSCLC. Examples of key oncogenes affected include *EGFR*, *RAS*, *PIK3CA*, and *AKT*. Conversely, examples of tumor suppressors that are inactivated include *PTEN* and *RASSF1*. **(B)** Genetic and epigenetic mechanisms responsible for the disruption of genes in the EGFR signaling pathway in NSCLC include DNA copy number alterations (amplification or deletion), point mutations, and DNA methylation changes. Thus, it is important to consider multiple aspects of the genome and epigenome simultaneously to elucidate the mechanisms driving pathway deregulation. *(This illustration was generated using Ingenuity Pathway Analysis software).*[38]

molecular-weight drugs that act intracellularly. Suppressor genes such as p53, genes controlling transcription factors such as MYC, and genes whose products have more challenging molecular characteristics such as RAS have turned out to be less "druggable." However, by knowing the upstream molecules that interact with these gene products, or the downstream molecules that they activate, targets may be identified that counteract the activity of these genes and are more "druggable."[40]

It was initially hoped that a drug which successfully targeted the product of an aberrant gene in one type of cancer would be effective in other types of cancer with the same genetic abnormality. However, this has not always turned out to be the case, because different types of cells can express pathways that may bypass the drug's target. An example is found with vemurafenib, which, as noted, is effective against the majority of melanomas expressing a mutated BRAF gene at V600E, but is not effective against colon cancers with the same genetic aberration.[41] This has been investigated by screening a panel of colon carcinoma cell lines bearing V600E RAF mutations with a short-hairpin RNA (shRNA) library representing the full complement of 518 human kinases, in the presence or absence of the BRAF inhibitor PLX4032. It was found that shRNA vectors targeting and eliminating EGF receptors converted resistant cells into becoming sensitive to inhibition by the BRAF inhibitor.[42] This effect was duplicated by treating cells with a drug or antibody against the EGF receptor. Thus inhibition of the EGF receptor tyrosine kinase appears to be required to permit sensitivity to this BRAF inhibitor in colorectal cancer cells. Most melanoma cells do not have the active EGF receptors that are seen in typical epithelial cells, and therefore are sensitive to the drug inhibiting BRAF. Clinical trials are testing this important experimental observation in patients with colorectal cancer bearing the V600E BRAF mutation, by adding an EGF receptor inhibitor to a BRAF inhibitor.

The above experiment is one example of many biological studies with cell lines and tumor biopsies that provide explanations for resistance to targeted drugs, involving activation of bypass pathways. For example, MET amplification can lead to resistance against the EGF receptor inhibitor gefitinib by activating ERB-B3.[43] Likewise, the efficacy of MEK inhibitors in basal-subtype breast cancer cells is limited by a feedback loop involving activation of the PI3K pathway by activation of the EGF receptor tyrosine kinase.[44] This suggests that a combination of inhibitors of MEK plus inhibitors of EGF receptors or the PI3K pathway may be effective in this situation.

In summary, combination treatments with agents against two different targets, in order to attack a driver gene and a bypass pathway concurrently, may provide a way to overcome resistance. Many clinical trials are testing this hypothesis.

With the technologies available today, an attractive way to investigate the mechanisms of failure (resistance) after initial response to an experimental targeted agent in a clinical trial is to biopsy the recurrent tumor and use genomic and expression array analysis—in comparison with the original tumor—to determine what new event or events have conferred resistance.

The bypass mechanisms that explain resistance to a targeted drug in a primary tumor may be discovered by direct comparison of genomic aberrations in primary tumor specimens from patients who are sensitive, or resistant, to a drug targeting a genetic aberration that they share. For example, clinical responses occur only in a minority of the colon cancer patients treated with the EGF receptor inhibitors cetuximab or panitumumab. It was found that colon cancers with a mutation in K-RAS were not responsive to these EGF receptor inhibitors.[45] Furthermore, when resistance to these anti-EGF receptor antibodies developed in colon cancer patients who were initially responsive, biopsy of the recurrence showed that a mutation in RAS (either from outgrowth of a minor subpopulation below the limits of detection in the primary tumor, or occurring de novo) accounted for resistance in the majority of patients.[46,47] Unfortunately, an effective inhibitor of K-RAS is not available to administer with the EGF receptor inhibitor. Because nearly 50% of colon cancers have mutations in K-RAS, screening for these mutations provides benefits to the patient by *avoiding* a therapy that will not be beneficial, and to controlling the costs of medical care by avoiding the use of these expensive agents in this clinical situation. Testing for this biomarker is now standard of care for treatment of colon cancer patients.

The complexity of the challenge in identifying genes that can modulate sensitivity and resistance to targeted therapies is exemplified by the many mechanisms of resistance that have been discovered in patients with malignant melanoma who were initially responsive to therapy with vemurafenib against V600E B-RAF, and subsequently relapsed. Six different mechanisms identified in tumor biopsies from these patients are listed in Table 45-2.[48-54] In all cases, confirmatory studies were carried out with cell lines containing the V600E B-RAF mutation, to demonstrate that the proposed

Table 45-2 Mechanisms of Resistance to Vemurafenib Treatment in Patients with V600E B-RAF Malignant Melanoma

Mechanism of Resistance	Reference
1. Mutation in MEK, downstream of B-RAF	48, 49
2. Increased copy number of V600E B-RAF	50
3. Aberrantly spliced, truncated v600E B-RAF	51
4. Upregulation of PDGF receptor B, or N-RAS mutation	52
5. PTEN loss reduces BIM-mediated apoptosis	53
6. Increased IGF-1R levels and activity	54

mechanism was able to confer resistance to the inhibitor. The common theme in this series of observations and in studies of resistance to other targeted therapies is that there are two predominant resistance mechanisms—new aberrations of the targeted gene and activation of bypass pathways.

These data naturally stimulate the formation of hypotheses on how to overcome resistance to drugs that target B-RAF. One approach that was tested recently was the addition of an inhibitor of MEK, administered concurrently with an inhibitor of mutated B-RAF. The rate of complete or partial response with the combination was 76%, as compared with 54% with monotherapy (P = 0.03).[55] Of course the hope is that these responses will be more durable, resulting in substantial prolongation of life—but the results will not be available for a few years.

Although driver genes and genes to which cancer cells are addicted are obvious targets for drug development, there are other genes and pathways that do not cause cancer but are important for the survival and growth of cancer cells. This has generated the concept of "non-oncogene addiction."[56] Examples include gene products involved in DNA damage repair, protein chaperones and heat shock proteins, histone modification, and buffering of reactive oxygen species. Attacking these pathways that support the malignant phenotype, in addition to targeting critical oncogenes, may be a fruitful approach to combination therapy of cancer.

The series of experiments just discussed demonstrate the appropriateness of the term *precision medicine*. This term describes the situation in which a more precise understanding of the various genetic aberrations in a particular patient's cancer, and their interactions with each other and with the surrounding microenvironment, can provide decision support to the treating oncologist—enabling selection of a targeted drug or drugs that are appropriate for that patient's illness. In this chapter, the emphasis has been on genomics as the basis of precision medicine. This is because at the present time, genomic biomarkers are the most useful for drug development and for making clinical decisions. In the future, it is anticipated that biomarkers from at least four other sources will contribute more and more to precision medicine:

- *Expression of genes*. Data are being reported suggesting that a considerable fraction of mutated genes are not expressed. In the case of suppression genes, this is expected, because this is the basis for their impact on the cell's phenotype. For potential oncogenes, however, this prevents the genetic aberrancy from having an impact. Furthermore, the level of expression of a gene—unrelated to the presence of a mutation—may alter cell behavior and phenotype. Again, the EGF receptor provides an example. This receptor is overexpressed (10- to 1000-fold) on a large fraction of epithelial cancers and is likely to create a "non-oncogene addiction" in these cells.[56] Thus an important area of cancer research will involve closely examining the expression of RNA and proteins coded for by aberrantly functioning genes, to assess the importance of their expression levels in identifying a biomarker signature useful for precision cancer care.
- *Epigenetics*. Current technologies and computational methods in this area are not adequate to inform clinical decisions. Many investigators believe that epigenetic changes play a major role in oncogenesis and the malignant phenotype.[57] This is a topic that will likely need to be visited more thoroughly in the future, as advancements are made in knowledge and assay techniques.
- *Immunologic profile*. As discussed in Chapters 50-52, immunological interventions are beginning to affect cancer therapy, leveraging recent gains in understanding the functioning of the human immune system. The patient's immune system is likely to play an important role in controlling malignancy, as was postulated five decades ago. With increased understanding of the role of cytokines derived from the patient's lymphocytes and leukocytes in promoting or inhibiting cancer cell growth, a profile of the patient's immune functions is likely to become a component of the biomarker profile informing precision cancer care.[58]
- *Proteomics*. Evaluation of the functional status of proteins in a patient's cancer (e.g., phosphorylation status) and detection of circulating protein markers derived from cancers (e.g., HCG levels) are promising areas for the clinic and should expand in value as biomarkers, as mass spectrometry techniques and the use of reverse phase protein arrays are developed into more useful clinical tests.[59]

Predicting Drug Efficacy and Toxicity

The other major scientific gap identified in the Report from the President's Council of Advisors on Science and Technology underscored the need for improved ways of predicting at the earliest possible stage of development the efficacy and toxicity of candidate drugs. This requires continued efforts at developing improved models of human cancer in cell culture and animal models.

At the level of cell culture, screening on arrays containing hundreds of human cancer cell lines is providing new data on mechanisms of drug action, on potential synthetic-lethal targets which predict pairs of targeted drugs that may be effective when either alone is not,[60] and on patterns of gene aberrations and gene expression that predict sensitivity or resistance to specific targeted experimental drugs.

At the level of drug-testing systems in animal models, progress has been made on two fronts:

- Growth of primary human tumor specimens in direct xenografts, without an intermediate step of culture on plastic surfaces, has produced models of human cancer in mice that are more closely comparable to primary human cancers.[61]
- GEMMs with inducible promoters on mutated genes permit activation of the aberrant gene in specific organ sites and at specific time points, creating murine models that can more closely mimic human cancers.[21]

Mouse models are discussed in Chapters 8 and 9.

Clinical Trials

The sequencing of clinical trials that is currently used to study new cancer drugs is presented in Chapter 48. There are two advances that may improve the efficiency and effectiveness of trials with experimental cancer therapies. The first is the increased use of adaptive randomization of patients to receive one of two drugs, or one from a panel of drugs. Initially, patients are assigned randomly to receive one or another of the drugs. As results accumulate, patients are assigned preferentially (but not invariably) to drugs that are producing more benefit. Eventually, with accrual of data on additional patients, poorly performing drugs are eliminated and new drugs can be added.[62] This method depends on powerful preset biostatistical tools that track and calculate probabilities of benefit sequentially, as results on each patient are added continuously to the database. The BATTLE Trial in advanced lung cancer, discussed earlier, was one of the first with targeted experimental therapies to use adaptive randomization, and the I-Spy 2 Trial for breast cancer is one of many current examples that is ongoing. This approach[63] is quite different from the standard randomization protocol, in which the number of patients to be studied is predetermined by a calculation of the number needed for statistical significance of the results. Patients are then assigned randomly to one of two or more arms of the trial, and results remain undisclosed until the trial reaches a predetermined point for evaluation. The advantage of adaptive randomization is the capacity to study more experimental agents and reach yes-or-no decisions with fewer patients, which speeds up the process and reduces the number of patients who are receiving a drug that is not going to be beneficial. However, there is concern about the possibility of moving too swiftly and discarding an experimental drug inappropriately because decisions are made on a small number of patients, which bypasses the value of randomizing large numbers in order to stratify for unknown variables that might affect responses.

The other major advance in clinical trial design involves more stringent criteria for selecting patients in the earliest Phase II setting, once the dose and toxicity issues are settled in Phase I. A number of the successful trials described in this chapter enrolled only patients whose cancers had been shown to bear the genetic aberration that the experimental drug was designed to target. It is possible that off-target effects of an experimental drug may make an important contribution to its efficacy. However, the logic of this approach to testing drugs that target the products of a specific aberrant gene is obvious, provided that the experimental drug does indeed work primarily by acting on that target. With this approach, fewer patients will have to be studied to reach statistically valid conclusions, drug approval by the FDA will be achieved in shorter time frames, and patients will receive treatment with a drug that is more likely to be effective. A positive result in a clinical trial performed in this manner validates the genetic aberration tested for in patients' cancers as a useful biomarker. The successes with this approach have led to Phase I/II trials, where biomarker-based enrollment in Phase II on a fixed dose and schedule is a seamless transition from Phase I.

As noted, most cancers are likely to have a number of driver genetic aberrations—a typical estimate is five—and it is not clear which or how many need to be targeted to eliminate the malignant cells. Because most drugs given at maximal tolerated doses only partially block their targets, and because of the redundancies in most signaling and regulatory pathways, it is likely that there are few occasions when a cancer cell is so totally addicted to a particular aberrant gene that a single drug targeting the product of that gene will cause death of the cancer cell. This reasoning, combined with knowledge of reported clinical outcomes data, suggest that optimal anticancer treatment is likely to require administration of drugs in combinations. In the past, combinations were selected based on the efficacy of individual drugs with nonoverlapping toxicities, whereas today a more rational selection of combinations of targeted drugs is based on knowledge of intersecting signaling and regulatory pathways and data on the genomic aberrations in more than one driver gene in a particular cancer.[64,65]

Bringing two or more investigational drugs into clinical trials for use in combinations has been a challenging task, because the FDA required demonstration of the efficacy and safety of each drug alone before a combination could be tested. The FDA has released a new Guidance Document that provides guidelines for exploring combinations more efficiently, in situations where one or both new drugs are unlikely to provide benefit to patients when administered alone.[66]

With the proliferation of data on the genomic and molecular aberrations in many thousands of patients' cancers and data on their responses to both targeted and conventional therapies, we will have new opportunities to learn by retrospectively correlating genomic and clinical data and comparing effectiveness of all available treatment options. The growing fields of health information technology and medical informatics should make this possible.[67,68] Examples of insights that can be gained include discovery of a rare toxicity of a cancer therapy and discovery of an unanticipated association between responsiveness to a drug and a particular signature of genomic aberrations. However, many issues remain to be solved. Perhaps the most daunting is the need for standardized nomenclature and formats for electronic reporting of data, and interoperability between multiple clinical databases from multiple sources. Large health care providers such as Kaiser Permanente have solved many of these problems with sophisticated information systems that process and analyze large amounts of clinical data. There should be a federated system of access to data that protects patient confidentiality, but allows wide access for data mining and analysis as well as hypothesis testing. Another factor that prevents data sharing is the desire of academic institutions, clinical researchers, and pharmaceutical companies to sequester data that they can control because of concerns about protection of intellectual property.

Once analysis of databases and results of research projects on genomic-based cancer treatments have produced results, this information must be gathered and organized in a way that supports clinical decision making. Doctors and patients need analytical tools that enable them to make decisions about cancer therapy that are informed and evidence-based. Only a learning system which gathers and organizes information in ways that are accessible, reliable, and rapid will be useful to the practicing oncologist.[60] This is a challenge for the future.

The implementation of genomics-based personalized cancer care raises a number of ethical issues: confidentiality of information; clarity on the level of certainty about clinical inferences that can be made from the results of tests for biomarkers; and the level of patient counseling appropriate for proper understanding of genomic data. Interpretation of genomic data is rendered difficult by intrinsic errors in the methodology, the high number of apparent aberrations observed in DNA from most cancer cells, the challenge of determining which genetic abnormality is a relevant driver of a patient's cancer, and the heterogeneity within each human cancer that can invalidate predictions based on data from only a single sampling of the tumor. Genomic data are not perfectly predictive, and both physicians and patients must understand and accept this in order to use the data appropriately to improve decisions about cancer therapy.

Table 45-3 Challenges for Personalized Cancer Therapy

1. Unresolved biological and molecular questions:
   - Tumor heterogeneity within a site and between sites
   - Identification of genetic aberrations that "drive" a cancer
   - Influence of tumor microenvironment
2. Complexity of clinical trial design and computational analysis of data
3. Requirement of an integrated process involving many clinical and research disciplines
4. A risk-averse "one at a time" clinical trial environment
5. Need for regulatory harmonization: FDA, CMS, CLIA, etc.
6. Need for decision support tools that supply actionable information to treating oncologists and their patients
7. Ethical concerns, especially related to privacy and control of data
8. Need for proof of value (benefit/costs) in order to justify reimbursements

CLIA, Clinical Laboratory Improvement Amendment; *CMS*, Centers for Medicare and Medicaid Services; *FDA*, U.S. Food and Drug Administration.

Many advances have been made, but many challenges remain to be addressed before personalized, precision treatment based on a more complete knowledge of a patient's cancer at the molecular level becomes the standard of practice. Table 45-3 lists some of these challenges.

Although much can be learned by sampling a patient's cancer for performance of genomic analysis, the procedure of obtaining a biopsy presents risks that must be taken into consideration, and biopsies cannot be performed repeatedly. Noninvasive ways of obtaining actionable information about a patient's cancer are desirable, and two that are being explored are promising. The most advanced noninvasive test for assessing molecular and metabolic properties of cancers involves medical imaging, especially positron emission tomographic (PET) scanning and magnetic resonance imaging (MRI).[69] The measurement of fluorodeoxyglucose (FDG) uptake with PET scanning to detect changes in glucose metabolism and measurement of deoxyfluorothymidine (FLT) uptake to estimate the rate of proliferation can help in the differential diagnosis of an abnormal mass and can provide a rapid demonstration of the effect of a therapy on cancer in a patient. Some very promising progress has been demonstrated in the imaging of gene products and molecular abnormalities in animal cancer models, and this work is in early stages of evaluation in the clinical setting.

The second noninvasive test that holds great promise is the evaluation of circulating cancer cells and circulating fragments of cancer cell DNA that are released into the blood.[70,71] The presence of circulating cancer cells and changes in their number have been shown to be predictive of prognosis and response to therapy in selected situations. DNA fragments are released into the circulation as a natural result of cell death in the body, and cancer cell DNA can be identified by the presence of genomic aberrations known to exist on the basis of prior assays of the patient's tumor. This may enable tracking of a patient's tumor burden by periodic sampling of DNA in the blood, as well as screening for a cancer recurrence or documenting a response to therapy.

It is hoped that these technologies will develop to the point where they can be advanced into standard practice during the next 5 to 10 years.

Conclusion

A striking lesson relevant to clinical oncology that comes from the expansion of research on human cancer during the past decade is the value of genetic and molecular biomarkers for identifying the most useful targets for new drug development, and for screening patients to identify those most likely to bear cancers that will respond to particular targeted therapies.

In parallel, advancements in understanding signaling and regulatory pathways in cells and their interactions in complex systems have enabled identification of targets for therapy that are most likely to be driving the behavior of cancer cells.

What is especially exciting is that knowledge and technology are enabling sophisticated laboratory research and diagnostic molecular assays to be performed on specimens of primary human cancers. The knowledge gained from those studies is more likely to be relevant to development and selection of effective therapies than the data from cell lines and xenografts, which we were forced to depend on for so many years.

Information that guides selection of therapy will continue to expand as the status of critical RNA and protein molecules is assayed on primary tumor specimens and metastases, and further understanding leads to measures of the patient's immune/inflammatory system as well as the influence of the microenvironment around the tumor.

The confluence of these approaches will lead to increasingly effective personalized care and precision medicine, moving us forward toward the ultimate goal of understanding how to give the right therapy to the right patient at the right time.

Key References

5. Mendelsohn J, Powis G. From bench to bedside with targeted therapies. In: Mendelsohn J, Howley PM, Israel MA, Gray JW, Thompson CB, eds. *The Molecular Basis of Cancer*. 3rd ed. Philadelphia, Pa: WB Saunders Co; 2008:521-530.
11. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J. Growth stimulation of A431 cells by EGF: Identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci USA*. 1983;80:1337-1341.
16. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-1037.
17. Divgi CR, Welt C, Kris M, et al. Phase I and imaging trial of indium-111 labeled anti-EGF receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. *J Natl Cancer Inst*. 1991;83:97-104.
18. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med*. 2004 Jul 22;351:3373-45.
19. Bishop JM. Cellular oncogenes and retroviruses. *Annu Rev Biochem*. 1983; 52:301-354.
20. Boehm JS, Hahn WC. Towards systematic functional characterization of cancer genomes. *Nat Rev Genet*. 2011 Jun 17;12:487-498.
21. Politi K, Pao W. How genetically engineered mouse tumor models provide insights into human cancers. *J Clin Oncol*. 2011 Jun 1;29:2273-2281.
22. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004 May 20;350:2129-2139.
23. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004 Jun 4;304: 1497-1500.
24. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004 Sep 7;101: 13306-13311.
25. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med*. 2009 Sep 3;361:947-957.
26. Von Hoff DD, Stephenson Jr JJ, Rosen P, et al. Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. *J Clin Oncol*. 2010 Nov 20;28:4877-4883.
27. Kim ES, Herbst RS, Wistuba II , et al. The BATTLE Trial: personalizing therapy for lung cancer. *Cancer Discov*. 2011;1:44-53.
28. Janku F, Tsimberidou AM, Garrido-Laguna I, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. *Mol Cancer Ther*. 2011;10:558-565.
29. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
30. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
31. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011 Mar 25;331:1553-1558.
32. Macconaill LE, Garraway LA. Clinical implications of the cancer genome. *J Clin Oncol*. 2010 Dec 10;28:5219-5228.
33. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. *Sci Transl Med*. 2011 Nov 30;3:111-121.
34. Rodón J, Saura C, Dienstmann R, et al. Molecular prescreening to select patient population in early clinical trials. *Nat Rev Clin Oncol*. 2012 Apr 3;9:359-366.
35. President's Council of Advisors on Science and Technology. *Report to the President Propelling Innovation in Drug Discovery, Development and Evaluation*, September 2012
36. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008 Sep 26;321:1801-1806.
37. Amit I, Garber M, Chevrier N, et al. Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. *Science*. 2009 Oct 9;326:257-263.
38. Vucic EA, Thu KL, Robison K, et al. Translating cancer 'omics' to improved outcomes. *Genome Res*. 2012;22:188-195.
39. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heal of cancer. *Science*. 2002 Jul 5;297:63-64.
40. Sellers WR. A blueprint for advancing genetics-based cancer therapy. *Cell*. 2011 Sep 30;147:26-31.
41. Kopetz S, Desai J, Chan E, et al. PLX4032 in metastatic colorectal cancer patients with mutant BRAF tumors. *J Clin Oncol*. 2010;28(suppl):15s (abstr 3534).
42. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012 Jan 26;483:100-103.

43. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007 May 18;316:1039-1043.
44. Mirzoeva OK, Das D, Heiser LM, et al. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. *Cancer Res*. 2009 Jan 15;69:565-572.
45. Van Cutsem E, Köhne CH, Lçng I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol*. 2011;29:2011-2019.
46. Hudson KL. Genomics, health care, and society. *N Engl J Med*. 2011;365:1033-1041.
47. Diaz Jr LA, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012 Jun 28;486:537-540.
48. Emery CM, Vijayendran KG, Zipser MC, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. *Proc Natl Acad Sci U S A*. 2009;106:20411-20416.
49. Wagle N, Emery C, Berger MF, et al. Dissecting THERAPEUTIC Resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol*. 2011 Aug 1;29:3085-3096.
50. Shi H, Moriceau G, Kong X, et al. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun*. 2012 Mar 6;3:1-8.
51. Poulikakos PI, Persaud Y, Janakiraman M, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF (V600E). *Nat Lett*. 2011 Dec 15;480:387-391.
52. Nazarian R, Shi H, Wang Q, et al. Melanomas require resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nat Lett*. 2010 Dec 16;468:973-979.
53. Paraiso KHT, Xian Y, Rebecca VW, et al. PTEN loss confers BRAF inhibitor resistance to melanoma cells through the suppression of BIM expression. *Cancer Res*. 2011;71:2750-2760.
54. Villaneuva J, Vultur A, Lee JT, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell*. 2010 Dec 14;18:683-695.
55. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367:1694-1703.
56. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell*. 2009 Mar 6;136:823-837.
57. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. 2011 Mar;17:330-339.
58. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*. 2010 Jun;29:309-316.
59. Hanash S, Taguchi A. The grand challenge to decipher the cancer proteome. *Nat Rev Cancer*. 2010 Sep;10:652-660.
60. Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*. 2005 Sep;5:689-698 Review.
61. Hidalgo M, Bruckheimer E, Rajeshkumar NV, et al. A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. *Mol Cancer Ther*. 2011 Aug;10:1311-1316.
62. Biswas S, Liu DD, Lee JJ, Berry DA. Bayesian clinical trials at the University of Texas M. D. Anderson Cancer Center. *Clin Trials*. 2009 Jun;6:205-216.
63. Barker AD, Sigman CC, Kelloff GJ, Hylton NM, Berry DA, Esserman LJ. I-SPY 2: an adaptive breast cancer trial design in the setting of neoadjuvant chemotherapy. *Clin Pharmacol Ther*. 2009 Jul;86:97-100.
64. Kummar S, Chen HX, Wright J, et al. Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. *Nat Rev Drug Discov*. 2010;9:843-856.
65. Al-Lazikani B, Banerji U, Workman P. Combinatorial drug therapy for cancer in the post-genomic era. *Nat Biotechnol*. 2012 Jul 10;30:679-692.
66. *Guidance for Industry Codevelopment of Two or More Unmarketed Investigational Drugs for Use in Combination*. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm
67. Institute of Medicine. *Informatics Needs and Challenges in Cancer Research: Workshop Summary*. Washington, DC: The National Academies Press; 2012 1-129.
68. Abernethy AP, Etheredge LM, Ganz PA, et al. Rapid-learning system for cancer care. *J Clin Oncol*. 2010 Sep 20;28:4268-4274.
69. Pysz MA, Gambhir SS, Willmann JK. Molecular imaging: current status and emerging strategies. *Clin Radiol*. 2010;65:500-516.
70. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008 Sep;14:985-990.
71. Maheswaran S, Haber DA. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev*. 2010 Feb;20:96-99.

References

1. de Kruif P. *Microbe Hunters*. New York, NY: Harcourt, Brace & Co; 1926:326.
2. Gilman A, Philips FS. The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. *Science*. 1946;103:409-436.
3. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid (Aminopterin). *N Engl J Med*. 1948;238:787-793.
4. Burchenal JH, Murphy ML, Ellison RR, et al. Clinical evaluation of a new anti-metabolite, 6-mercaptopurine, in the treatment of leukemia and allied diseases. *Blood*. 1953;8:965-999.
5. Mendelsohn J, Powis G. From bench to bedside with targeted therapies. In: Mendelsohn J, Howley PM, Israel MA, Gray JW, Thompson CB, eds. *The Molecular Basis of Cancer*. 3rd ed. Philadelphia, Pa: WB Saunders Co; 2008:521-530.
6. Frei 3rd E, Freireich EJ. Progress and perspectives in the chemotherapy of acute leukemia. *Adv Chemother*. 1965;2:269-298.
7. Li MC, Whitmore Jr WF, Golbey R, Grabstald H. Effects of combined drug therapy on metastatic cancer of the testis. *JAMA*. 1960 Nov 5;174:1291-1299.
8. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.
9. Miller RA, Maloney DG, Warnke R, Levy R. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Engl J Med*. 1982 Mar 4;306:517-522.
10. Huggins C. Endocrine-induced regression of cancers. *Science*. 1967;156:1050-1054.
11. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J. Growth stimulation of A431 cells by EGF: Identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci USA*. 1983;80:1337-1341.
12. Gill GN, Kawamoto T, Cochet C, et al. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem*. 1984;259:7755-7760.
13. Masui H, Kawamoto T, Sato JD, Wolf B, Sato GH, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-EGF receptor monoclonal antibodies. *Cancer Res*. 1984;44:1002-1007.
14. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol*. 1989 Mar;9:1165-1172.
15. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
16. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med*. 2001;344:1031-1037.
17. Divgi CR, Welt C, Kris M, et al. Phase I and imaging trial of indium-111 labeled anti-EGF receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. *J Natl Cancer Inst*. 1991;83:97-104.
18. Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med*. 2004 Jul 22;351:3373-45.
19. Bishop JM. Cellular oncogenes and retroviruses. *Annu Rev Biochem*. 1983;52:301-354.
20. Boehm JS, Hahn WC. Towards systematic functional characterization of cancer genomes. *Nat Rev Genet*. 2011 Jun 17;12:487-498.
21. Politi K, Pao W. How genetically engineered mouse tumor models provide insights into human cancers. *J Clin Oncol*. 2011 Jun 1;29:2273-2281.
22. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004 May 20;350:2129-2139.
23. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004 Jun 4;304:1497-1500.
24. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A*. 2004 Sep 7;101:13306-13311.
25. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med*. 2009 Sep 3;361:947-957.
26. Von Hoff DD, Stephenson Jr JJ, Rosen P, et al. Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. *J Clin Oncol*. 2010 Nov 20;28:4877-4883.
27. Kim ES, Herbst RS, Wistuba II , et al. The BATTLE Trial: personalizing therapy for lung cancer. *Cancer Discov*. 2011;1:44-53.
28. Janku F, Tsimberidou AM, Garrido-Laguna I, et al. PIK3CA mutations in patients with advanced cancers treated with PI3K/AKT/mTOR axis inhibitors. *Mol Cancer Ther*. 2011;10:558-565.
29. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
30. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
31. Stratton MR. Exploring the genomes of cancer cells: progress and promise. *Science*. 2011 Mar 25;331:1553-1558.
32. Macconaill LE, Garraway LA. Clinical implications of the cancer genome. *J Clin Oncol*. 2010 Dec 10;28:5219-5228.
33. Roychowdhury S, Iyer MK, Robinson DR, et al. Personalized oncology through integrative high-throughput sequencing: a pilot study. *Sci Transl Med*. 2011 Nov 30;3:111-121.
34. Rodón J, Saura C, Dienstmann R, et al. Molecular prescreening to select patient population in early clinical trials. *Nat Rev Clin Oncol*. 2012 Apr 3;9:359-366.
35. President's Council of Advisors on Science and Technology. *Report to the President Propelling Innovation in Drug Discovery, Development and Evaluation*, September 2012
36. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008 Sep 26;321:1801-1806.
37. Amit I, Garber M, Chevrier N, et al. Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. *Science*. 2009 Oct 9;326:257-263.
38. Vucic EA, Thu KL, Robison K, et al. Translating cancer 'omics' to improved outcomes. *Genome Res*. 2012;22:188-195.
39. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heal of cancer. *Science*. 2002 Jul 5;297:63-64.
40. Sellers WR. A blueprint for advancing genetics-based cancer therapy. *Cell*. 2011 Sep 30;147:26-31.
41. Kopetz S, Desai J, Chan E, et al. PLX4032 in metastatic colorectal cancer patients with mutant BRAF tumors. *J Clin Oncol*. 2010;28(suppl):15s (abstr 3534).
42. Prahallad A, Sun C, Huang S, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012 Jan 26;483:100-103.
43. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science*. 2007 May 18;316:1039-1043.
44. Mirzoeva OK, Das D, Heiser LM, et al. Basal subtype and MAPK/ERK kinase (MEK)-phosphoinositide 3-kinase feedback signaling determine susceptibility of breast cancer cells to MEK inhibition. *Cancer Res*. 2009 Jan 15;69:565-572.
45. Van Cutsem E, Köhne CH, Lçng I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol*. 2011;29:2011-2019.
46. Hudson KL. Genomics, health care, and society. *N Engl J Med*. 2011;365:1033-1041.
47. Diaz Jr LA, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012 Jun 28;486:537-540.
48. Emery CM, Vijayendran KG, Zipser MC, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. *Proc Natl Acad Sci U S A*. 2009;106:20411-20416.
49. Wagle N, Emery C, Berger MF, et al. Dissecting THERAPEUTIC Resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol*. 2011 Aug 1;29:3085-3096.

50. Shi H, Moriceau G, Kong X, et al. Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun*. 2012 Mar 6;3:1-8.
51. Poulikakos PI, Persaud Y, Janakiraman M, et al. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF (V600E). *Nat Lett*. 2011 Dec 15;480:387-391.
52. Nazarian R, Shi H, Wang Q, et al. Melanomas require resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nat Lett*. 2010 Dec 16;468:973-979.
53. Paraiso KHT, Xian Y, Rebecca VW, et al. PTEN loss confers BRAF inhibitor resistance to melanoma cells through the suppression of BIM expression. *Cancer Res*. 2011;71:2750-2760.
54. Villaneuva J, Vultur A, Lee JT, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell*. 2010 Dec 14;18:683-695.
55. Flaherty KT, Infante JR, Daud A, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med*. 2012;367:1694-1703.
56. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell*. 2009 Mar 6;136:823-837.
57. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. *Nat Med*. 2011 Mar;17:330-339.
58. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev*. 2010 Jun;29:309-316.
59. Hanash S, Taguchi A. The grand challenge to decipher the cancer proteome. *Nat Rev Cancer*. 2010 Sep;10:652-660.
60. Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer*. 2005 Sep;5:689-698 Review.
61. Hidalgo M, Bruckheimer E, Rajeshkumar NV, et al. A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. *Mol Cancer Ther*. 2011 Aug;10:1311-1316.
62. Biswas S, Liu DD, Lee JJ, Berry DA. Bayesian clinical trials at the University of Texas M. D. Anderson Cancer Center. *Clin Trials*. 2009 Jun;6:205-216.
63. Barker AD, Sigman CC, Kelloff GJ, Hylton NM, Berry DA, Esserman LJ. I-SPY 2: an adaptive breast cancer trial design in the setting of neoadjuvant chemotherapy. *Clin Pharmacol Ther*. 2009 Jul;86:97-100.
64. Kummar S, Chen HX, Wright J, et al. Utilizing targeted cancer therapeutic agents in combination: novel approaches and urgent requirements. *Nat Rev Drug Discov*. 2010;9:843-856.
65. Al-Lazikani B, Banerji U, Workman P. Combinatorial drug therapy for cancer in the post-genomic era. *Nat Biotechnol*. 2012 Jul 10;30:679-692.
66. *Guidance for Industry Codevelopment of Two or More Unmarketed Investigational Drugs for Use in Combination*. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm
67. Institute of Medicine. *Informatics Needs and Challenges in Cancer Research: Workshop Summary*. Washington, DC: The National Academies Press; 2012 1-129.
68. Abernethy AP, Etheredge LM, Ganz PA, et al. Rapid-learning system for cancer care. *J Clin Oncol*. 2010 Sep 20;28:4268-4274.
69. Pysz MA, Gambhir SS, Willmann JK. Molecular imaging: current status and emerging strategies. *Clin Radiol*. 2010;65:500-516.
70. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008 Sep;14:985-990.
71. Maheswaran S, Haber DA. Circulating tumor cells: a window into cancer biology and metastasis. *Curr Opin Genet Dev*. 2010 Feb;20:96-99.

William N. Hait, Eric Rubin, and Joseph R. Bertino

46 Cancer Therapeutics

Cancer is characterized by the transformation of normal cells to ones characterized by abnormal cellular differentiation, proliferation, invasion, and metastases. The molecular and biochemical bases underlying the transformation process are becoming increasingly clear and provide critically important information for identifying new drug targets.

Normal cell division results from the interaction of growth factors with specific receptors (plasma membrane, cytoplasmic, nuclear). This initiates a signal transduction cascade through receptor tyrosine kinases and downstream serine, threonine kinases that culminates in uncoiling of DNA by the action of histone acetylases and topoisomerases and activation of nuclear transcription factors that produce cell-proliferation and cell-viability molecules. It should not, therefore, be surprising that cancer cells usurp these normal pathways and that our most effective drugs target many of these processes (Figure 46-1).

Malignant cells acquire the ability to replicate indefinitely, invade, and metastasize. This process includes activation of telomerase, detachment from the primary site, anchorage-independent growth, invasion through the basement membrane, and access to the blood or lymphatic vessels, as well as entry to distant organs through adherence to visceral capillaries and the ability to grow in a foreign site, thereby escaping a variety of immunological mechanisms designed to protect the host from "foreign invasion." An understanding of the interactions between cancer cells and the surrounding stroma (malignant tissue) helped identify new targets to interfere with this characteristic of malignancy.

Recent attention has been turned to the existence and role of cancer stem cells. Dick and colleagues first identified cells from acute myeloid leukemia that had stem-cell characteristics[1] based on the identification of a subpopulation of cells with a $CD34^+/CD38^-$ phenotype that possessed the ability to recapitulate the phenotypic heterogeneity of the original leukemia. Subsequently, cancer stem cells (CSCs) or tumor-initiating cells have been found in other leukemias and in most solid tumors.[2] The therapeutic importance of the stem cell model is that it posits that our inability to cure most tumors is due to resistance of CSCs to current chemotherapy and radiation therapy.

In this chapter, we attempt to place cancer chemotherapeutic drugs in a molecular biological context as summarized in Tables 46-1, 46-2, and 46-3.*

Molecular Basis of the Therapeutic Index

Therapeutic index is defined as follows: LD_{50}, or *median lethal dose*, is the dose of drug that causes death in 50% of experimental animals, and ED_{50}, or *median effective dose*, is the dose that produces a specified effect ("response") in 50% of the population under study. The therapeutic index in the clinic compares the dose of a drug that causes untoward toxicities to the dose that produces the desired therapeutic effect.

All drugs have targets, but it is the unique relationship of the target to the disease that can ultimately affect a drug's therapeutic index. Traditional drug targets included DNA (nucleotide bases, enzymes of DNA synthesis, degradation, and repair), microtubules, and growth factor receptors. New targets include mutated, overexpressed, or fused growth factor/oncogene products (EGFR [Her-1], Her-2/neu, ras, bRaf, bcr:abl), immune checkpoint modulators (CTLA4, PD-1), cell surface antigens (CD33, CD22, CD20), anti-apoptotic proteins (bcl-2), cell-cycle regulators (cyclin-dependent kinases), epigenetic targets (histone deacetylases and methyltransferases), metabolic pathways (mTOR, PI3K, AKT) , stem-cell pathways (notch, wnt), and the machinery

*We focus primarily on drugs that have achieved approval from the U.S. Food and Drug Administration (FDA); numerous drugs against new targets are in development, but a comprehensive description is beyond the scope of this chapter.

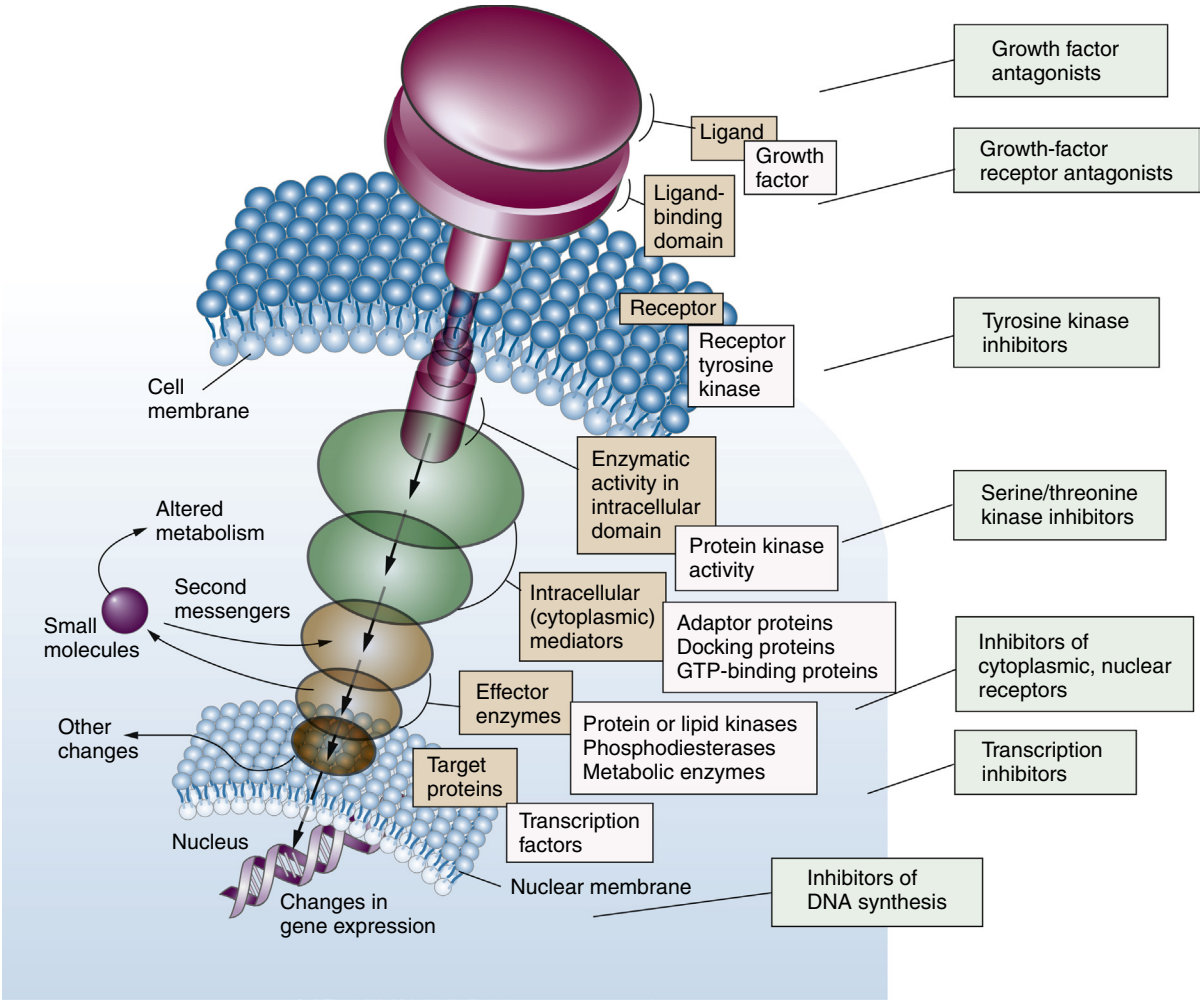


Figure 46-1 Targets for anticancer drugs Anticancer drugs work by interfering with the processes underlying normal cellular physiology. These include receptor-activated signal transduction pathways culminating in transcriptional activation, DNA replication, protein synthesis, and cell division. *(Modified by permission from Macmillan Publishers Ltd: Downward J. The ins and outs of signaling.* Nature. *2001;411:759).*

of protein synthesis (L-asparaginase) and degradation (proteasome). Drugs that affect these newer targets are often referred to as *targeted therapies*, creating the false impression that classic chemotherapeutics do not have targets.

The specificity/selectivity of a drug for a particular target is, in general, proportional to the affinity constant, K_a, more accurately defined for competitive drug target interactions as the K_i, which is the reciprocal of the concentration of drug required to inhibit 50% of the target's activity when controlled for all possible ligand or substrate concentrations. The activity of a drug refers to its effectiveness independent of dose or concentration.

There are several factors that help explain why cancer cells are more sensitive to cancer therapeutic drugs than normal tissues. For any drug, the therapeutic index is related to absorption, uptake, distribution, and metabolism. Differences in tumor vasculature, intratumoral pressure, and drug binding may alter drug uptake in a favorable or unfavorable way. Classically, the therapeutic index of intravenously administered cancer chemotherapy has been thought to be due primarily to cell-cycle kinetics. Many chemotherapeutic agents are more effective against cycling than noncycling cells and are tested under cell culture conditions where cancer cells rapidly proliferate ("log phase"); this a posteriori conclusion was derived from these observations. However, many solid tumors have a relatively long doubling time, yet a therapeutic index remains. Therefore, alternative explanations must exist. One includes differences in energy requirements between normal and malignant cells. For example, whereas normal tissues use oxidative phosphorylation to metabolize glucose, malignant tissues are often dependent on aerobic glycolysis. This is thought to reflect the selection pressure placed on tumor cells to cope with relatively hypoxic and nutrient-deprived conditions. Rather than using the electron transfer chain within the mitochondria to yield 36 mol ATP per mol glucose, cancer cells metabolize glucose via glycolysis, generating a net 2 mol ATP per mol glucose metabolized

Table 46-1 Chemotherapeutic Drug Overview

Target	Examples	Use
Nuclear Receptors		
Estrogen receptor	Tamoxifen, toremifene, raloxifene, fulvestrant	Treatment and prevention of breast cancer
Progesterone receptor	Megestrol acetate	Breast cancer
Retinoid receptor	Retinoic acid	Promyelocytic leukemia
Androgen receptor	Bicalutamide, flutamide, nilutamide, enzalutamide	Prostate cancer
Plasma Membrane Receptors and Tyrosine Kinases		
Her-1 (EGFR)	Gefitinib, erlotinib, cetuximab, panitumumab	Non–small-cell lung; pancreas
Her-2/neu	Trastuzumab, pertuzumab	Breast
Bcr:abl	Imatinib, dasatinib, nilotinib	Chronic myelogenous leukemia
VEGF receptor	Sorafenib, sunitinib	Renal cell
cKit	Imatinib, sorafenib, sunitinib	Gastrointestinal stromal tumor (GIST)
Flt-3	Sorafenib, sunitinib	
Fms	Sunitinib	
Gonadotropin receptors	Abarelix, leuprolide, goserelin	
Growth factors		
Aromatase inhibitors Cyp17A1	Letrozole, anastrozole, exemestane Abiraterone acetate	Breast cancer Prostate cancer
Vascular endothelial growth factor (VEGF)	Bevacizumab	Colorectal cancer
Multiple Kinases		
VEGFR, PDGFR, cKIT, Flt-3, FMS, bRAF, Ret	Sunitinib	Renal; GIST
VEGFR, PDGFR, cRAF, bRAF, Flt-3, Fms	Sorafenib	Renal
Miscellaneous		
CD20 CD25	Rituximab, ibritumomab tiuxetan (Zevalin), tositumomab (Bexxar), ofatumumab Denileukin diftitox	Lymphoid malignancies Cutaneous T-cell lymphoma
CD52 CD30	Alemtuzumab Brentuximab vedotin	Chronic lymphocytic leukemia (CLL) Hodgkin's disease
DNA Synthesis		
Dihydrofolate reductase	Methotrexate, trimetrexate	Breast, lymphocytic leukemia, choriocarcinoma, lymphoma
Thymidylate synthase	5-Fluorouracil, capecitabine, pemetrexed	Colon, breast, mesothelioma
Adenosine deaminase	Pentostatin, cladribine (2CDA)	Hairy-cell leukemia, lymphomas, CLL
DNA Replication		
Nucleic acid bases		
Alkylating agents	Nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, bendamustine)	Leukemia, lymphoma, breast, brain, melanoma, etc.
	Nitrosoureas (e.g., BCNU, temozolomide)	Glioblastoma, anaplastic astrocytoma
	Ethyleneimines (e.g., thiotepa)	Bone marrow transplantation
	Alkyl sulfonates (e.g., busulfan)	Leukemia
	Triazenes (e.g., dacarbazine)	Melanoma
Platinating agents	Cis-, carbo-, oxaliplatin	Lung, head and neck, bladder, germ cell, colorectal

Continued

Table 46-1 Chemotherapeutic Drug Overview—cont'd

Target	Examples	Use
Transcription inhibitors	Actinomycin-D	Wilms' tumor
DNA methylation	5′-Azacytidine, decitabine	Myelodysplastic syndrome
Topoisomerases		
Topoisomerase I	Topotecan, irinotecan	Colorectal, ovary, lung, cervical
Topoisomerase II	Doxorubicin, epirubicin, etoposide, mitoxantrone	Breast, lymphoma, leukemia, lung, ovary, testicular, etc.
Microtubules		
Vinca alkaloids	Vincristine, vinblastine, vinorelbine	Breast, lung, acute lymphocytic leukemia (ALL), bladder, lymphoma, etc.
Taxanes Epothilones	Paclitaxel, docetaxel Ixabepilone	Breast, lung, bladder, ovarian, etc. Breast cancer
Protein Synthesis		
	L-Asparaginase	Childhood ALL, T-cell lymphoma
Protein Degradation		
60S proteasome	Bortezomib, carfilzomib	Multiple myeloma, mantle-cell lymphoma

Table 46-2 Drugs That Alter Nucleic Acid Synthesis and Function

Target	Examples	Use
DNA Synthesis		
Dihydrofolate reductase	Methotrexate, trimetrexate, pemetrexed, pralatrexate	Breast, lymphocytic leukemia, choriocarcinoma, lymphoma, etc.
Thymidylate synthase	5-Fluorouracil, capecitabine, pemetrexed	Colon, breast, mesothelioma, etc.
Adenosine deaminase	Pentostatin, cladribine (2CDA)	Hairy-cell leukemia, lymphomas, CLL
DNA Replication		
Nucleic acid bases		
Alkylating agents	Nitrogen mustards (e.g., mechlorethamine, cyclophosphamide)	Leukemia, lymphoma, breast, brain, melanoma, etc.
	Nitrosoureas (e.g., BCNU)	
	Ethyleneimines (e.g., thiotepa)	
	Alkyl sulfonates (e.g., Busulfan)	
	Triazenes (e.g., dacarbazine, temozolomide)	
Platinating agents	Cis, carbo-, oxaliplatin	Lung, head and neck, bladder, germ cell, colorectal
Transcription inhibitors	Actinomycin-D	Wilms' tumor
DNA methylation	5′-Azacytidine	Myelodysplastic syndrome
Topoisomerases		
Topoisomerase 1	Topotecan, irinotecan	Colorectal, ovary, lung, cervical
Topoisomerase II	Doxorubicin, epirubicin, etoposide, mitoxantrone	Lymphoma, leukemia, lung, ovary, testicular

(the Warburg effect). As a result, cancer cells are metabolically fragile and are unable to cope as readily with cellular damage. This may be particularly relevant for drugs that block the effects of growth factors, because growth factor depletion produces rapid downregulation of nutrient transporters, which would lead to metabolic crisis in cancer cells more rapidly than in normal cellular counterparts.

Another concept is that tumor cells exhibit "oncogene addiction" and that inhibition of one or more of these oncogene products rapidly results in apoptotic cell death in

Table 46-3 Drugs with Other Mechanisms

Target	Examples	Use
Microtubules		
Vinca alkaloids	Vincristine, vinblastine, vinorelbine	Breast, lung, ALL, bladder, lymphoma
Taxanes	Paclitaxel, docetaxel	Breast, lung, bladder, ovarian
Protein Synthesis		
	L-Asparaginase	Childhood ALL, T-cell lymphoma
Protein Degradation		
60S proteasome	Bortezomib	Multiple myeloma, mantle-cell lymphoma

"addicted" cancer cells but not in normal counterparts. Thus, tumor cells that depend on their survival by overexpression of a growth factor receptor (e.g., EGFR) would be more susceptible to inhibitors than are normal cells.

Drugs Affecting Growth Factors and Growth Factor Receptors

Cancer cells usurp the mechanisms of normal cell division, which results from the interaction of growth factors with specific receptors (plasma membrane, cytoplasmic, or nuclear). This initiates a signal transduction cascade culminating in activation of nuclear transcription factors that produce cell-proliferation and cell-viability molecules. Thus, it stands to reason that some of our most effective drugs target growth factors or their receptors (see Table 46-1) and the downstream consequences of this interaction that include activation of protein kinases, replication of DNA, transcription of mRNA, synthesis of new proteins, formation of the mitotic spindle through microtubule polymerization, and creation of interphase daughter cells via microtubule depolymerization (see Table 46-2). In addition, the nutrient requirements of cancer cells create an increased dependence on the uptake of glucose (the basis for positron emission tomography or PET scan) and the ability to sustain energy requirements through frequent alterations in the PI3K/AKT/mTOR pathway and activation of autophagic cell survival (Figure 46-2).

Drugs Affecting Growth Factors

Sex Hormones

Aromatase is an enzyme complex made up of two proteins, aromatase cytochrome P450 (CYP19) and NADPH-cytochrome P450 reductase. Inhibition of aromatase blocks the conversion of androgens (androstenedione) to estrone in peripheral tissues including fat, liver, muscle, and breast without detectable effects on adrenal synthesis of corticosteroids or aldosterone. Following the reports by Santen and colleagues that aminoglutethimide could inhibit the conversion of androstenedione to estradiol,[3] aromatase became an attractive target for new drug development. Three aromatase inhibitors are used in the clinic, including letrozole (Femara), anastrozole (Arimidex), and exemestane (Aromasin). Whereas letrozole and anastrozole are reversible, nonsteroidal inhibitors of aromatase, exemestane is a steroidal derivative of androstenedione that binds irreversibly to the enzyme and targets the protein for degradation. Aromatase inhibitors further deplete circulating estradiol in postmenopausal women and are highly effective in the treatment of breast cancer in the adjuvant and metastatic settings. These well-tolerated medications can produce osteopenia and are often prescribed with a bisphosphonate, calcium, and vitamin D to prevent this complication.

Cyp17A1 is a cytochrome P450 enzyme complex that has both hydroxylase and lyase activity. It catalyzes the hydroxylation of pregnenolone and progesterone to their 17-OH derivatives and the conversion of 17-hydroxyprogesterone and 17-hydroxypregnenolone to DHEA and androstenedione via its lyase activity, leading to the synthesis of androgenic steroids in the gonads, adrenals, fat, and tumor stroma. Abiraterone acetate (Zytiga) is a prodrug of abiraterone, a potent and selective Cyp17 inhibitor shown to increase overall survival in patients with castration-resistant prostate cancer.[4]

Gonadotropin-Releasing Hormones

Drugs that target receptors for gonadotropin-releasing hormones (GnRHs) decrease the production of ovarian or testicular hormones. The most widely used agents (leuprolide, goserelin) are agonists of GnRH receptors that cause an immediate increase in gonadotropins and eventually produce castration levels of sex hormones by the desensitization of GnRH receptors. A newer agent, abarelix (Plenaxis), is a GnRH receptor antagonist that immediately decreases GnRHs without the disadvantage of an initial hormone surge.

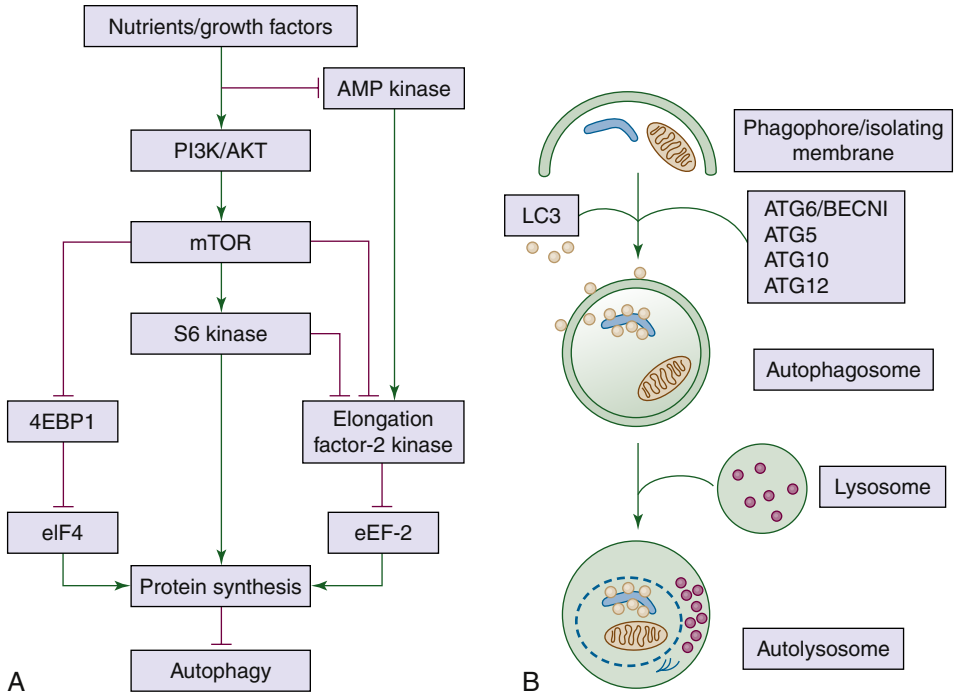


Figure 46-2 Energy regulation through PI3 kinase signaling Cancer cells require exquisite control of energy utilization because of a dependence on aerobic glycolysis to generate ATP. The PI3 kinase pathway serves as an energy sensor with links to self-preservation through autophagic cell survival. **(A)** When there are adequate nutrients, the pathway is activated, protein synthesis is increased, and autophagy is inhibited. In contrast, in the case of nutrient deprivation, protein synthesis is suppressed and autophagy is activated. **(B)** The process of forming the autophagosome in response to nutrient deprivation. *(From Moreau P, Pylypenko H, Grosicki S. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study.* Lancet Oncol. *2011;12:431-440).*

Vascular Endothelial Growth Factor

The observation by Folkman and colleagues that tumors stimulate blood vessel formation, coupled with the knowledge that angiogenesis is required for tumor growth and metastasis, led to a search for effective inhibitors of this process.[5] Vascular endothelial growth factor (VEGF) is produced by normal and neoplastic cells and regulates angiogenesis. The demonstration by Kim and colleagues[6] that a murine monoclonal antibody against VEGF had preclinical activity led to the development of *bevacizumab*, a human monoclonal antibody that binds to and inhibits VEGF, preventing its interaction with VEGF receptors (Flt-1 and KDR) that are present on the surface of endothelial cells,[7] thus inhibiting endothelial cell proliferation.

Bevacizumab (Avastin) was first approved for first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil–based chemotherapy and now has several other indications. Its most common side effects include hypertension, thrombosis, and proteinuria, but asthenia, gastrointestinal perforation, wound dehiscence, hemorrhage, and nephrotic syndrome have been reported, as has been a possible increase in congestive heart failure. Recently, a recombinant fusion protein, *ziv-aflibercept* (Zaltrap), referred to as a "VEGF trap," has been developed and approved in combination with FOLFIRI for refractory colorectal cancer. The VEGF trap fuses the extracellular domain of VEGF receptors 1 and 2 with an Fc domain of human IgG1, thereby acting as a soluble receptor for VEGFs.[8]

Drugs Affecting Growth Factor Receptors

Steroid Hormone Receptors

The interaction between steroid hormones and intracellular receptors recruits co-activators and co-repressors to the nuclear transcription complex, leading to transcriptional activation of genes containing specific steroid response elements.

Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) bind with high affinity to cytoplasmic and nuclear estrogen receptors, then recruit transcriptional co-activators and co-repressors to the transcription complex, where they bind to estrogen response elements within promoter regions of estrogen-regulated genes. In certain tissues SERMs are antiestrogenic, whereas in others they are estrogenic; this is believed to be due to differential recruitment of co-repressors versus repressors in a specific tissue type. For example, *tamoxifen* (Nolvadex) behaves as an estrogen receptor antagonist in breast tissue, but as an agonist in the uterus, bone, and

liver. As a result, tamoxifen is a highly effective drug for the treatment of breast cancers that express hormone receptors but has the disadvantage of also increasing endometrial proliferation and the risk of uterine cancer. The estrogenic effect of tamoxifen on bone prevents osteopenia, while its estrogenic effects on the liver lower cholesterol and increase the incidence of thrombosis. In contrast, *raloxifene* (Evista) is a SERM that behaves as an antiestrogen in breast, uterus, and liver, but retains estrogenic activity in the bone. Although results with raloxifene were disappointing for the treatment of metastatic breast cancer, it is effective for breast cancer prevention.[9] *Fulvestrant* (Faslodex) is a pure antiestrogen that binds to the estrogen receptor and targets it for ubiquitin-mediated proteasomal degradation.

Enzalutamide (MDV 3100)[1] is a potent and selective androgen receptor antagonist that has recently been shown to increase survival in patients with castration-resistant prostate cancer.[10]

Bexarotene (Targretin) is an analog of vitamin A that binds with high affinity to retinoid "X" receptors. It is approved for use against refractory cutaneous T-cell lymphoma. Its side effects include cheilitis, headache, myalgias, and arthralgias, as well as an increase in liver function tests, hypertriglyceridemia and hypercalcemia, and hypothyroidism.

Drugs that Affect Oncogenic Growth Factor Pathways

Epidermal Growth Factor Receptor Family

Her-2/Neu. The discovery of a transforming element in the DNA of a malignant glial cell line by Weinberg and colleagues led to the identification of HER-2/neu.[11] When Slamon's group observed that HER-2/neu was overexpressed in aggressive breast cancers, a search ensued for a means to inhibit this membrane receptor.[12] The biology of HER-2/neu activation is complex with both putative and proven ligands. Intracellular signaling occurs after receptor activation that leads to hetero- and homodimerization with other members of the EGFR receptor family, including HER-1, HER-3, and HER-4.

Trastuzumab. Trastuzumab (Herceptin) is approved for use alone or in combination with paclitaxel for the treatment of metastatic breast cancers that overexpress HER-2/neu. In the 25% to 30% of patients who respond to treatment, a substantial number survive for long periods of time. Unfortunately, these patients relapse, often in the central nervous system (CNS), probably because of the inability of trastuzumab to cross the blood-brain barrier rather than a predilection of these cells for the CNS. Trastuzumab is also effective in breast cancer patients receiving adjuvant chemotherapy.[13]

Her-1 (*epidermal growth factor receptor [EGFR]*). EGFR is overexpressed in 60% to 80% of colorectal cancers and in many other tumor types. It can be transforming in laboratory models. The demonstration by Mendelsohn and colleagues that antibodies directed against the EGFR extracellular domain inhibited the growth of cancer cells led to the development of several types of EGFR antagonists.[14] Cetuximab (Erbitux) is a humanized mouse monoclonal antibody used in the treatment of *Kras* wild-type colorectal cancer alone or in combination with irinotecan, and more recently in combination with the FOLFOX regimen. Cetuximab has activity in head and neck cancers and is synergistic with radiation in preclinical models. Cetuximab is well tolerated. The major side effects include asthenia and an acneiform rash.

Several drugs have been developed to target the tyrosine kinase domain of the EGFR. For example, gefitinib (Iressa) received accelerated U.S. Food and Drug Administration (FDA) approval for the treatment of refractory non–small-cell lung cancer (NSCLC), but failed to show a survival advantage in larger randomized clinical trials. In contrast, erlotinib (Tarceva), a structurally similar molecule, produced a survival advantage in patients with NSCLC and is approved for use in this group of patients. Two reports demonstrated that gefitinib was particularly active in patients whose tumors harbored activating mutations in the tyrosine kinase catalytic domain of EGFR.[15,16] This alteration was particularly prevalent in nonsmokers, women of Japanese descent, and tumors of bronchoalveolar histology. Erlotinib also received approval in combination with gemcitabine for the treatment of pancreatic cancer. Acneiform rash and diarrhea are the most common side effects of these generally well-tolerated medications.

Bcr:abl. Of all the newer "targeted" therapies, none has been more impressive than imatinib (Gleevec; 4-[(4-methyl-1-piperazinyl)methyl]-*N*-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate) for use in chronic myelogenous leukemia, gastrointestinal stromal tumors, and a chronic myeloproliferative disease characterized by eosinophilia.[17] Imatinib targets the tyrosine kinase domain of the fusion protein formed by the reciprocal translocation involving the long arms of chromosomes 9 and 22 [t(9;22)(q34.1;q11.21)], referred to as the *Philadelphia chromosome* (Figure 46-3). Imatinib is also active against the tyrosine kinase activity of c-kit and the platelet-derived growth factor receptor (PDGFR), the former accounting for its activity against GIST and the latter for its activity against the chronic myeloproliferative syndrome. *Denileukin diftitox*, or *DAB389IL-2 (ONTAK)*, is a fusion protein consisting of cytotoxic A and B chain fragments (Met[1]-Thr^{387}-His) of diphtheria toxin fused to interleukin-2 (IL-2).[18] It is approved for the treatment of persistent or recurrent cutaneous T-cell lymphoma expressing the CD25 component of

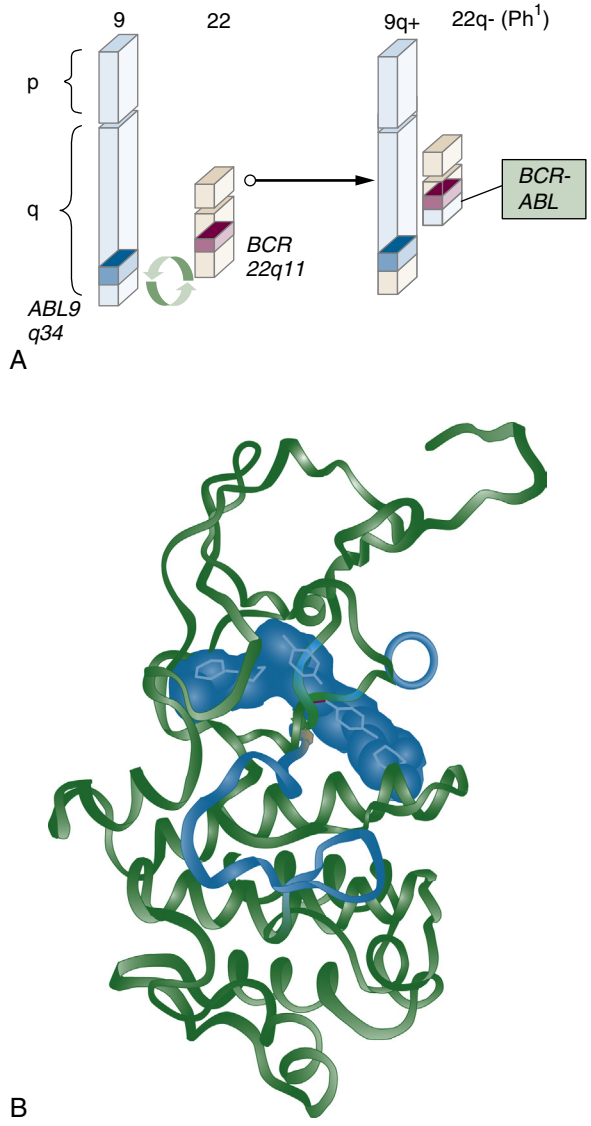


Figure 46-3 Genetic and structural changes underlying the activity of imatinib **(A)** The 9:22 chromosomal translocation that produces the bcr/abl oncogenic tyrosine kinase. **(B)** Ribbon drawing of the structure of the Abl kinase domain *(green)* in complex with imatinib (Gleevec). The activation loops and the van der Waals surfaces corresponding to the inhibitor are colored. Helix α C and the interlobe connector are shown in *dark green.* *(From Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia.* N Engl J Med. *2002;346:645-652).*

the IL-2 receptor. Denileukin thereby delivers this potent exotoxin, which ADP ribosylates elongation factor-2 and terminates protein synthesis. Side effects include flulike symptoms, acute hypersensitivity reactions, nausea and vomiting, vascular leak syndrome, infections, and transient elevation of liver function tests.

bRaf. Sequencing of the melanoma genome revealed a *bRaf* mutation in approximately 60% that replaced a valine with a negatively charged glutamic acid, creating constitutive activation of the enzyme. *Vemurafenib* (Zelboraf) selectively inhibits the mutant form of the kinase. In a 55-patient dose escalation trial, Flaherty and co-workers[19] demonstrated that among 32 patients with the bRaf(V600E) mutation, 24 had PRs and 2 had CRs for an 81% objective response rate (ORR). A notable adverse event was the appearance of cutaneous squamous-cell carcinomas, mostly of the low-grade keratoacanthoma variety. Resistance appears to emerge rapidly because of NRAS mutation, PDGFR upregulation, and overexpression of the map kinase COT, which suggests ways of further improving results in the future.

Drugs that target multiple signal transduction enzymes. Signaling in malignant tissues is often complex. Therefore drugs that target a single pathway may not be as effective as those that have multiple targets. Protein kinase inhibitors are rarely completely selective for a given target, given the similarities in the catalytic domains of these enzymes. Two drugs have recently been approved that have relatively permissive activities (i.e., they inhibit multiple kinases including those that phosphorylate tyrosines, serines, or threonines in substrate proteins). For example, *sorafenib* (Nexavar) was approved for the treatment of renal-cell carcinoma and was initially believed to be specific for bRaf kinase, a serine/threonine kinase downstream of Ras that is mutated in melanoma and activated in several other malignancies. However, sorafenib is also a potent inhibitor of the VEGF receptor tyrosine kinases VEGFR-2 and -3, FLT-3, Kit, and PDGFR-β.[20] Similarly, *sunitinib* (Sutent), which was recently approved for treatment of renal-cell carcinoma and for GIST patients who have progressed following treatment with imatinib, can also inhibit multiple kinases including PDGFR-α and -β, VEGFR-1, -2, and -3, Kit, FLT-3, CSF-1, and RET.[21] These drugs possess a different spectrum of untoward side effects from that of classical cytotoxic agents. For example, hypertension, bleeding (including tumor hemorrhage), diarrhea, mucositis, skin rash, and taste abnormalities occur more frequently than for placebo. Sorafenib was associated with hand-foot syndrome (palmer plantar dysesthesia), and both drugs appear to increase cardiac events. Patients receiving sunitinib were more likely to have decreases in their left ventricular ejection fraction (10%) than placebo-treated controls (1%), and those on sorafenib were more likely to experience cardiac ischemic events (2.9%) than controls (0.4%).

Crizotinib (Xalkori). Fewer than 6% of NSCLCs harbor a fusion of EML4 with ALK. Crizotinib, an MTKi originally developed for the inhibition of cMET, also is active against ALK. This led to the rapid demonstration that crizotinib was highly effective for this subset of patients who rarely harbor *ras* or *EGFR* abnormalities.[22] In a disease with a historical response rate of about 10%, they observed a response rate to crizotinib of 57% and a disease control rate of 90%. ALK-positive patients tend to be younger, light or never smokers, and have a histology

characterized by abundant mucin production and the presence of signet ring cells.

Drugs That Target Cancer Stem Cells

Like normal marrow, skin and colon stem cells, CSCs are relatively resistant to chemotherapy and radiation. Mechanisms of resistance include an increase in drug efflux pumps, an increase in DNA repair capacity, and aberrant or overexpression of key signaling pathways such as hedgehog and notch. *Vismodegib* (Erivedge), an inhibitor of hedgehog signaling, has been found useful to treat basal-cell carcinoma, a malignancy characterized by upregulation of this pathway.[23] Inhibitors of the notch pathway are also being tested in the clinic, in particular when upregulation of this pathway can be found, as in T-cell leukemia.

Drugs That Target the Immune System

The observation that melanoma can be immunogenic, as manifested by spontaneous regression and vitiligo, focused attention on directing an immune response against this disease. The pioneering work of Rosenberg and colleagues demonstrating that activation of cytotoxic T cells by interleukin-2 could produce dramatic responses[24] led to the approval of *aldesleukin* (Proleukin) for the treatment of melanoma and renal-cell carcinoma.

CTLA4. Allison and colleagues hypothesized that inhibiting the function of immune inhibitory Treg cells would be an effective treatment for patients with advanced melanoma. Hodi and co-workers randomized 676 patients who were progressing on therapy for metastatic disease to *ipilimumab* (Yervoy), a monoclonal antibody targeting CTLA, alone or in combination with the gp100 protein and measured overall survival (OS) as the primary endpoint. Unlike most other therapies, ipilimumab significantly improved OS and ORR.[25] This result, however, came at the cost of autoimmune reactivity in the skin, gastrointestinal tract, and endocrine system and rare drug-related deaths (7 of 540). As physicians have gained experience with this form of treatment and learned how to manage these untoward effects, the safety profile is improving.

Programmed death 1[2] (PD-1). PD-1 is a cell surface receptor on T cells that functions to dampen immune response to certain antigens. Recent evidence suggests that targeting this receptor with an anti–PD-1 antibody (BMS-936558) can restore immune reactivity, resulting in tumor responses in melanoma, NSCLC, and renal-cell carcinoma.[26] Serious drug-related adverse events occurred in approximately 14% of patients.

Drugs That Alter Nucleic Acid Synthesis and Function

Growth factor/growth factor receptor interactions activate signal transduction cascades that initiate DNA synthesis through transcriptional activation of cell proliferation genes, culminating in DNA replication and cell division. A good example is phosphorylation of the retinoblastoma protein (Rb) by CDK4 and CDK6 followed by CDK2. This releases a family of bound transcription factors, E2F(s), which activate genes critical for progression into S phase and are overexpressed in malignancies (e.g., thymidylate synthase, dihydrofolate reductase). For DNA to then be transcribed, it must be made accessible to transcription factors. This is accomplished by releasing DNA from histone packaging via histone acetylases and unwinding of the double helical structure via the action of helicases and topoisomerases. Therefore, it is not surprising that some of our most effective chemotherapeutic drugs target these downstream events, including DNA synthesis, transcription, topoisomerase, and histone deacetylase and methylase activities.

Inhibitors of Nucleic Acid Synthesis

Dihydrofolate reductase (DHFR), the enzyme that replenishes reduced folate pools, was one of the earliest targets for cancer chemotherapy drugs that included *aminopterin* and *methotrexate*. By inhibiting DHFR, methotrexate and its polyglutamated derivatives deplete reduced folates and thereby block the synthesis of thymidylate and de novo purine synthesis. Similarly, targeting thymidylate synthase (TS) to interfere with DNA synthesis led to the development of 5-fluorouracil and 5-fluorodeoxyridine; these drugs remain critically important in the modern oncologist's armamentarium.

Pralatrexate (Folotyn) is a second-generation antifolate that also targets dihydrofolate reductase.[27] Compared to methotrexate, this compound is transported more efficiently into tumor cells via the reduced folate carrier and has better retention because it is a better substrate for polyglutamate synthetase, the enzyme that adds glutamates to reduced folates and folate analogs. In patients with relapsed or refractory peripheral T-cell lymphoma, a Phase II study showed a 29% response rate and duration of response of 10.1 months. Because patients with this disease have limited treatment options, it was approved by the FDA in 2009. It has subsequently been shown to be effective in patients with refractory

or relapsed cutaneous T-cell lymphoma, with response rates of 30% to 40%. The dose-limiting toxicity to this drug is stomatitis rather than marrow suppression. Pretreatment of patients with folic acid and vitamin B_{12} can ameliorate this toxicity, presumably without affecting antitumor effects.

Pemetrexed (Alimta) is a unique antifol containing a 6-5 fused pyrrolo[2,3,-*d*]pyrimidine nucleus that inhibits thymidylate synthase, glycinamide ribonucleotide formyltransferase (GARFT), and dihydrofolate reductase, folate-dependent enzymes involved in the synthesis of thymidine and purine nucleotides. Like methotrexate, pemetrexed is transported into cells by the reduced folate carrier and membrane folate-binding proteins, where it is metabolized to polyglutamates by folylpoly-gamma-glutamate synthetase. Polyglutamated forms are retained intracellularly and have greater affinity for TS and GARFT than pemetrexed monoglutamate. Pemetrexed also inhibits DHFR. Therefore, this drug interrupts de novo synthesis of thymidine and purine nucleosides.[28] Pemetrexed is approved for the treatment of mesothelioma and NSCLC. Pretreatment with folic acid and vitamin B is now used to ameliorate the most frequent side effects, including bone marrow suppression, fatigue, and skin rash.

Capecitabine (Xeloda) is an orally administered carbamate derivative of 5′-deoxy-5-fluorouridine that acts as a prodrug of 5-FU.[29] It is approved for use as a single agent in metastatic breast cancer that is resistant to anthracyclines and taxanes and in combination with docetaxel for metastatic breast cancer after relapse from anthracyclines. It is also approved as a single agent in the first-line treatment of metastatic colorectal cancer. Capecitabine is converted to 5′-deoxy-5-fluorocytidine (DFCR) by carboxylesterases in the liver. DFCR is then converted to 5′-deoxy-5-fluorouridine (DFUR) by cytidine deaminase in the liver and tumor tissue. DFUR is then converted to 5-FU by thymidine phosphorylase. The therapeutic index of capecitabine may be based on an increased activity of thymidine phosphorylase in tumor compared to normal tissues; therefore, capecitabine may have additional selectivity over 5-FU, although this has not been rigorously demonstrated in the clinic. Its side effects—neutropenia, diarrhea, stomatitis, and palmar-plantar dysesthesia (hand-foot syndrome)—are similar to those seen when 5-FU is given by continuous intravenous infusion.

Gemcitabine (Gemzar) is the 2′-deoxy-2′,2′-difluorocytidine analog of deoxycytidine, which was selected for development because of its activity against murine solid tumors.[30] It is approved for the treatment of recurrent pancreatic cancer and for front-line treatment of inoperable, locally advanced, or recurrent/metastatic NSCLC as well as breast and ovarian cancer.

Gemcitabine inhibits DNA synthesis by intracellular conversion by deoxycytidine kinase to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides that lead to competitive inhibition of DNA polymerase. In addition, gemcitabine diphosphate inhibits ribonucleotide reductase, thereby blocking the synthesis of deoxynucleoside triphosphates for DNA synthesis. Gemcitabine triphosphate also competes with dCTP for incorporation into DNA. The reduction in the intracellular concentration of dCTP (by the action of the diphosphate) enhances the incorporation of gemcitabine triphosphate into DNA (self-potentiation). Side effects include myelosuppression, nausea, vomiting, transaminitis, diarrhea, stomatitis, proteinuria, hematuria, fever, maculopapular rash, peripheral edema, and flu-like symptoms.

Clofarabine (Clolar) is a purine nucleoside antimetabolite approved for treating relapsed or refractory acute lymphocytic leukemia (ALL) in children after at least two other types of treatments have failed.[31] Efficacy and safety were demonstrated in a single multicenter trial in patients aged 2 to 19. Six patients (12%) achieved a complete remission (CR), 4 patients (8%) achieved a complete remission without total platelet recovery, and 5 patients (10%) achieved a partial response. Of the 15 responding patients, 6 had post-clofarabine bone marrow transplantation. The principal clofarabine toxicities were nausea, vomiting, and marrow suppression with febrile neutropenia. Clofarabine can produce systemic inflammatory response syndrome/capillary leak syndrome, manifested by the rapid development of tachypnea, tachycardia, hypotension, shock, and multiorgan failure. It can also cause left ventricular systolic dysfunction and tachycardia.

Inhibitors of DNA Topoisomerase

Topoisomerases correct the altered DNA topology that occurs during DNA replication and transcription by inflicting transient single-strand (topoisomerase I) or double-strand (topoisomerase II) breaks in DNA. Several natural-product antineoplastic drugs inhibit topoisomerase I or topoisomerase II. The effect of drugs on topoisomerases is different from that on most other enzyme inhibitors (i.e., they "poison" the enzymes by inhibiting religation of the DNA nicks produced during topoisomerase catalysis, thereby locking the enzyme in the "on" or catalytic conformation).[32]

Topoisomerase activity is increased during S phase, and cancer cells appear to have greater topoisomerase activity than their normal counterparts. Thus topoisomerase-targeting drugs inflict greater drug-induced DNA damage and cell death on cancer cells than on normal cells. Differences in the processing of topoisomerase-mediated DNA damage by malignant versus normal cells may also be important in the therapeutic index of these drugs.

Currently approved topoisomerase II inhibitors such as doxorubicin, daunomycin, mitoxantrone, etoposide, and

teniposide are part of many combination chemotherapy regimens and form the basis for some of our earliest curative regimens (e.g., non-Hodgkin lymphoma [doxorubicin], acute myelogenous leukemia [daunorubicin], and germ-cell malignancies [etoposide]). Additional topoisomerase II formulations include liposomal doxorubicin (Doxil), originally approved in 1995 for Kaposi sarcoma (Doxil is now approved for relapsed ovarian cancer) and *epirubicin* (Ellence), an anthracycline that is approved as a component of adjuvant therapy for node-positive breast cancer.

Currently approved topoisomerase I inhibitors include the camptothecin derivatives topotecan and irinotecan. Camptothecin was identified in the early 1960s by Wani and Wall as a potent anticancer alkaloid present in extracts from *Camptotheca acuminata* (the Chinese yew tree).[33] The sodium salt entered clinical trials in the 1970s but was discontinued because of severe and unpredictable toxicities. The appreciation of its unique mechanism of action by the Liu laboratory[32] and the pH dependence of lactone ring cleavage led to the development of stabler and safer derivatives. *Topotecan* (Hycamtin), a semisynthetic analog of camptothecin produced by adding a basic side chain at the 9-position of the A-ring of 10-hydroxycamptothecin, has increased water solubility without requiring hydrolysis of the lactone (E-ring). Topotecan was the first topoisomerase I inhibitor approved for clinical use and is indicated for refractory ovarian cancer and "sensitive" small-cell lung cancer after first-line chemotherapy. Its major untoward side effects include myelosuppression, fatigue, moderate nausea and vomiting, diarrhea, and alopecia.

Irinotecan (Camptosar; CPT-1) is another semisynthetic analog of camptothecin approved in 1996 for the treatment of colorectal cancer refractory to 5-FU. It is a prodrug that is converted to the active compound (SN-38) by carboxylesterases, which are present at relatively high concentrations in the intestine. Indications now include first-line treatment in combination with 5-FU and leucovorin for metastatic colon or rectal cancer and for colorectal cancer that has progressed or reoccurred following initial treatment with 5-FU. Its myeloid and gastrointestinal toxicities are enhanced when given in combination with 5-FU and leucovorin. Patients with Gilbert's disease or other polymorphisms in the UGT1A1 glucuronyl transferase have an increased risk of toxicity.[34] The combination of irinotecan and 5-FU/LV was approved in combination with bevacizumab for first-line treatment of metastatic colorectal cancer.

Alkylating Agents

DNA replication requires that the bases be accessible for Watson-Crick pairing. This normal biochemistry is interrupted by the alkylating agents.[35] The work of Alfred Gilman and Louis Goodman and their colleagues at Yale University demonstrated that nitrogen mustard could abolish lymphomas in experimental animals and had chemotherapeutic effects in man. These studies, carried out during World War II, were the start of cancer chemotherapy. The classes of alkylating agents include the nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, bendamustine), nitrosoureas (e.g., BCNU), ethyleneimines (e.g., thiotepa), alkyl sulfonates (e.g., busulfan), and the triazines (e.g., dacarbazine, temozolomide).

Temozolomide (Temodar) is the 3-methyl derivative of mitozolomide, which was discovered when screening a series of 1,2,4-triazenes and triazinones synthesized in the 1960s and 1970s.[36] Mitozolomide was the most promising compound, but it had severe and unpredictable side effects. Temozolomide, like DTIC, methylates N-7 and O-6 of guanine to produce the cytotoxic lesion. Temozolomide is approved for the treatment of adult patients with refractory anaplastic astrocytoma (disease progression after nitrosourea or procarbazine) and as adjuvant treatment when given with radiation to patients with glioblastoma multiforme. The most common side effects are lymphocytopenia, transaminitis, nausea and vomiting, hyperglycemia, anemia, and thrombocytopenia.

Bendamustine (Treanda) was used extensively to treat lymphoma in the 1980s in East Germany, but only became available in the United States in 1990.[37] It was approved by the FDA in 2008 for the treatment of CLL and indolent B-cell non-Hodgkin lymphoma that progressed during or within 6 months of treatment with rituximab or a rituximab-containing regimen. It acts as an alkylating agent causing intrastrand and interstrand crosslinks between DNA bases. The role of the purine ring in the molecule is not clear. It is usually used in combination with rituximab for the treatment of CLL and lymphoma. Common adverse reactions include nausea, fatigue, vomiting, diarrhea, fever, constipation, loss of appetite, cough, headache, unintentional weight loss, difficulty breathing, rashes, and stomatitis, as well as immunosuppression, anemia, and low platelet counts.

Platinating Agents

The therapeutic properties of cisplatin were deduced from studies of bacterial motility in electric currents, where bacterial cell death was observed adjacent to platinum electrodes.[38] Platinating agents consist of platinum complexed with ligands that are displaced by nucleophilic attack to produce inter- and intrastrand DNA adducts. Currently approved platinating agents include cisplatin, carboplatin, and oxaliplatin, which differ in their spectrum of activity and untoward side effects. For example, cisplatin is active

against lung cancer, head and neck cancer, and in combination with vinblastine and bleomycin led to the first reliable cures of testicular cancer. Cisplatin is highly emetogenic and is a potent oto- and nephrotoxin. In contrast, carboplatin appears to be less active than cisplatin against tumors of the head and neck and perhaps NSCLC. It is less emetogenic and nephrotoxic than cisplatin, but more myelotoxic. *Oxaliplatin* (Eloxatin; *trans*-l-diaminocyclohexane oxalatoplatinum) is a divalent coordination complex of platinum consisting of an oxalato group and a 1,2-diaminocylohexane (DACH) ligand. This third-generation platinum derivative is more active than cisplatin against colon cancer. Unlike cisplatin or carboplatin, renal dysfunction, ototoxicity, and alopecia are uncommon. The dose-limiting toxicity is peripheral neuropathy, which can be acute (lasting less than 14 days) or persistent (14 days or greater); an acute syndrome of pharyngolaryngeal dysesthesia can be exacerbated by exposure to cold (temperature, objects, or liquids). Oxaliplatin is approved for the treatment of colorectal cancer in either the adjuvant or metastatic settings. The activity of oxaliplatin is dependent on the formation of intrastrand (Pt)-DNA adducts/crosslinks, similar to that of other Pt compounds. DNA crosslinks inhibit replication and transcription and activate apoptosis. Oxaliplatin has been reported to also downregulate TS and increase sensitivity to fluoropyrimidines.[39] Oxaliplatin forms 2- to 10-fold fewer Pt-DNA adducts than cisplatin at equimolar and equitoxic concentrations, suggesting that these adducts may be more cytotoxic or that alternative mechanisms of action exist. DACH-Pt-DNA adducts formed by oxaliplatin are bulkier and more hydrophobic than *cis*-diamine-Pt-DNA adducts and may have greater inhibitory effects on DNA repair. DNA mismatch repair complexes do not recognize DACH-Pt-DNA adducts.

Epigenetic Modulators

The modification of DNA sequences by methylation and acetylation provides a remarkably diverse and powerful control over gene expression. The work of Peter Jones, Steve Baylin, and many others highlighted the types of epigenetic modifications that appear to be part of malignant transformation.[40] Furthermore, drugs that alter these epigenetic "marks" through enzyme inhibition of histone methyltransferases, such as *decitabine* (Dacogen) and *azacitidine* (Vidaza), or histone deacetylases, such as *suberoylanilide hydroxamic acid* (SAHA; vorinostat, Zolinza) and romidepsin, have had some success in the clinic.

Romidepsin. Romidepsin, isolated from a culture of *Chromobacterium violaceum*, was found to have cytotoxic effects against several human cancer cell lines in vitro and against xenografts. It was subsequently found to inhibit histone deacetylase.[41] Romidepsin acts as a prodrug, with the disulfide bond undergoing reduction within the cell to release a zinc-binding thiol. The thiol reversibly interacts with a zinc atom in the binding pocket of Zn-dependent histone deacetylase to block its activity. The FDA approved romidepsin for treatment of peripheral T-cell lymphoma (PTCL) in 2011 based on a Phase II study that showed a 25% response rate with 15% of patients achieving a CR.[40] The median duration of response was 17 months. The most common side effects were nausea and vomiting, fatigue, infection, loss of appetite, and anemia, thrombocytopenia, and leukopenia.

Vorinostat (SAHA, suberoylanilide hydroxamic acid) is a potent histone deacetylase inhibitor approved by the FDA for treatment of refractory cutaneous T-cell lymphoma based on a large Phase II study.[42] Response and duration of responses were similar to romidepsin. Side effects include fatigue, diarrhea, nausea, hyperglycemia, and thrombocytopenia.

Drugs that Affect the Mitotic Apparatus

Vinca Alkaloids, Taxanes, and Epothilones

Microtubules are essential for normal cellular function. They form the mitotic spindle, maintain cell shape, organize the location of organelles, mediate intracellular transport and secretion, and neurotransmission as well as axonemal flow and cell motility. Microtubules are composed of α- and β-tubulin dimers organized in bundles of 13 protofilaments that form hollow cylinders. The protofilaments are aligned with the same polarity. The (+) or fast-growing end moves outward from the nucleus to the plasma membrane, whereas the (−) or slow-growing end marks the site of nucleation of the microtubule, which often begins in the centrosome. Time-lapse microscopy has demonstrated that microtubules grow in spurts or may disappear altogether. This process, termed *dynamic equilibrium*, is an essential feature of microtubule physiology. For microtubules to elongate they require the addition of both α- and β-tubulin bound to GTP. Once bound to GTP, β-tubulin forms a GTP cap at the elongating end. Rapid microtubule growth requires bound GTP to increase the affinity for other tubulin molecules. During depolymerization, GTP is hydrolyzed more rapidly than it can be added, resulting in weakening of the bonds that hold the tubulin molecules together.

Antimitotic drugs act by interfering with the normal dynamic equilibrium of microtubules, thereby disrupting the function of the mitotic apparatus. In addition, by affecting microtubules in interphase cells, these drugs inhibit cell motility and normal subcellular organization.

Drugs Affecting Microtubule Polymerization

Paclitaxel (Taxol) was isolated in 1971 by Wani and Wall as an active moiety from the bark of the pacific yew, *Taxus brevifolius*.[43] The taxanes are large alkaloid esters consisting of a taxane ring linked to a four-member oxetan ring at positions C-4 and C-5. Docetaxel (Taxotere), a semisynthetic derivative produced from 10-deacetylbaccatin III, is more water soluble and more potent in vitro. Initially, the difficulties encountered in formulating this insoluble compound and its toxicities in patients diminished enthusiasm for developing this new agent. However, interest in paclitaxel was renewed when the Horowitz laboratory identified its unique mechanism of action (i.e., stabilization of polymerized microtubules[44]). Paclitaxel and docetaxel share broad-spectrum antitumor activity including breast, lung, ovarian, and bladder cancers. Both have effects against lymphoid malignancies. Nab-paclitaxel (Abraxane) is a derivatized formulation of paclitaxel bound to albumin nanoparticles, allowing the drug to be administered at a higher maximum tolerated dose and without Cremophor-El, thereby eliminating the need for extensive premedication. The albumin moiety may help target this agent to tumors expressing SPARC, the albumin receptor.[45]

Taxanes preferentially bind to the N-terminal 31 amino acids of the β-subunit of tubulin oligomers or polymers and inhibit microtubule depolymerization. At nanomolar concentrations, the taxanes produce a mitotic block without increasing microtubule polymer mass. At stoichiometric concentrations (1 M drug per 1 M tubulin dimer), taxanes polymerize and stabilize microtubules in the absence of GTP or microtubule-associated proteins (MAPs); these microtubules are resistant to depolymerization by calcium or low temperature.

The taxanes have broad-spectrum anticancer activity. They are used predominantly in the treatment of solid tumors (ovarian, breast, lung, bladder, head, and neck). Although docetaxel is more potent than paclitaxel, there is little direct evidence that it is more effective. The combination of docetaxel with estramustine or prednisone prolongs survival of patients with hormone-refractory prostate cancer.[46] Preclinical studies demonstrated that unlike most chemotherapeutic agents, taxanes are more active against cancer cells harboring p53 mutations; this may help explain their widespread activity and therapeutic index.[47] Both paclitaxel and docetaxel produce peripheral neuropathy, dose-limiting bone marrow suppression, and alopecia. Nausea, vomiting, and diarrhea occur with both drugs but are rarely severe. Docetaxel can cause vascular permeability (peripheral edema, pleural effusions, and ascites). Fluid retention occurs at cumulative doses above 400 mg/m^2 and may be decreased by lower single doses (less than 60 to 75 mg/m^2) or premedication with dexamethasone. Docetaxel also produces skin toxicities including an erythematous maculopapular rash of the forearms and hands. Both paclitaxel and docetaxel cause type I hypersensitivity reactions characterized by flushing, bronchospasm, dyspnea, and hypotension.

Ixabepilone (Ixempra) is a member of the epothilone family of natural product macrolides initially discovered as cytotoxic metabolites from the myxobacterium *Sorangium cellulosum*. They have a similar mechanism of action to the taxanes and compete for a binding site on polymerized microtubules.[48]

Drugs Affecting Microtubule Depolymerization

The vinca alkaloids were identified as extracts from the pink periwinkle plant (*Catharanthus roseus* G. Don.) that produced granulocytopenia in rats. This observation led to the isolation of four active alkaloids, of which two, vincristine and vinblastine, became active therapeutic agents. Today, this class also includes vinorelbine and vindesine.

The vinca alkaloids are large symmetrical molecules consisting of a dihydroindole nucleus (vindoline) connected to an indole nucleus (catharanthine) by a methylene bridge. Vincristine and vinblastine differ by a single R1 substituent, whereas vinblastine and vindesine differ in the R2 and R3 positions; vinorelbine has a modification of the catharanthine ring.

The mechanism of action of these drugs is concentration related. At substoichiometric concentrations, they bind to high-affinity sites at the ends of microtubules (K_a 5.3 × 10^{-5} M) and prevent microtubule polymerization. At higher concentrations, vincas bind to low-affinity, high-capacity sites (K_a 3-4 × 10^{-3} M) and lead to the disintegration of formed microtubules.

Despite structural similarities, the spectrum of activity and toxicities of the vinca alkaloids are different. For example, vincristine is highly effective against non-Hodgkin lymphoma, Hodgkin's disease, and pediatric solid tumors, yet vincristine has little activity against adult solid tumors. In contrast, vinorelbine is active against breast and lung cancer. Vinblastine is most frequently used in the treatment of testicular cancer and non-Hodgkin lymphoma and is an active agent in the treatment of breast cancer. Vinca alkaloids are potent inhibitors of angiogenesis, which may contribute to their activity.[49] Major toxicities include dose-limiting myelosuppression and neurotoxicity. Vinblastine and vinorelbine produce far greater neutropenia than vincristine, with nadirs occurring at 4 to 10 days with recovery seen in most patients by 7 to 21 days. All three agents cause mild alopecia and are severe vesicants. Vinorelbine may cause chest pain and other deep-seated pain of unspecified origin. Respiratory reactions include acute bronchospasm and subacute cough;

dyspnea and pulmonary infiltrates have also been reported and appear responsive to steroids. The most frequent neurotoxicities are numbness and tingling of the extremities, loss of deep tendon reflexes, and distal muscle weakness. Although the sensory changes are bothersome, they usually reverse over time and may not require discontinuation of the drug. Loss of motor function is a later and more ominous side effect, requiring discontinuation of the medication and or a search for other contributing factors.

Brentuximab vedotin is a chimeric anti-CD30 antibody conjugated to four molecules of a potent tubulin inhibitor, monomethyl auristatin.[50] This antibody-drug conjugate targets CD 30, a surface protein fairly specific to Reed-Sternberg cells and anaplastic large-cell lymphoma (ALCL). Key to the success of brentuximab vedotin is the cleavable dipeptide linker that is stable in serum, but once internalized is cleaved by lysosomes releasing auristatin. This drug was approved in 2011 for the treatment of relapsed Hodgkin's lymphoma patients and of systemic ALCL after failure of at least one multiagent chemotherapy regimen. The response rate in Hodgkin's patients with refractory disease was 75%, with 34% complete remissions. In ALCL there was an 86% response rate and 53% complete remissions. Similar to other antitubulin agents, brentuximab vedotin causes peripheral neuropathy, with 52% experiencing neuropathy of any grade, and 8% grade 3 or 0% grade 4 neuropathy. The neuropathy, usually sensory, can also be motor and irreversible; patients need to be monitored carefully during treatment. Other side effects include marrow suppression, fatigue, and gastrointestinal effects.

Drugs That Affect Protein Synthesis and Degradation

Few drugs affecting protein synthesis and/or degradation have been used for the treatment of cancer. This is somewhat surprising given that the complexity of the process provided important targets for the development of antibacterial antibiotics. The process of protein synthesis includes initiation, elongation, and termination requiring an array of amino acids, structural proteins, enzymes, and substrates including ribosomes, initiation factors, elongation factors, termination factors, and protein kinases that regulate the function of many of these elements. Historically, L-asparaginase has been the prototype of this class of drug. Recent attention has turned to the factors that control protein degradation. The ansamycins (e.g., geldanamycin) represent a class of agents that interfere with protein chaperones (i.e., proteins that bind to and stabilize newly formed or damaged proteins). Proteins targeted for degradation due to damage, improper folding, or cellular excess may proceed through one of two pathways, lysosomal or proteasomal. Bortezomib (Velcade), a drug that broadly inhibits proteases that are present in the proteasome, has been approved for the treatment of refractory myeloma.[51] It interferes with proteasomal degradation of proteins such as the NFκB inhibitor, IκB. NFκB is a constitutively activated transcription factor in myeloma[52] shown to promote oncogenesis by increasing growth factors (IL-6, VEGF), cellular adhesion molecules (ICAM1, VCAM1), and antiapoptotic proteins (bcl-2, IAP). For a drug that interferes with a fundamental cellular process, bortezomib is relatively well tolerated. Peripheral neuropathy is the most troublesome side effect, which can be ameliorated by subcutaneous administration.[53]

Recent attention has focused on the PI3K/AKT signaling pathway because it is frequently altered during malignant transformation. This pathway provides exquisite control over energy utilization (see earlier discussion and Figure 46-3) by regulating protein synthesis,[54] the most voracious energy consumer. Inhibitors of mTOR such as everolimus (Afinitor) and PI3Kδ are recent examples of this class of agent.

Key References

2. Vira D, Basak SK, Veena MS, et al. Cancer stem cells, micro RNAs, and therapeutic strategies including natural products. *Cancer Metastasis Rev*. 2012;31:733-571.
4. de Bono JS, Logothetis CJ, Molina A, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*. 2011;364:1995-2005.
5. Folkman J. Angiogenesis. *Annu Rev Med*. 2006;57:1-18.
8. Bagnasco L, Piras D, Parodi S, et al. Role of angiogenesis inhibitors in colorectal cancer: sensitive and insensitive tumors. *Curr Cancer Drug Targets*. 2012;12:303-315.
9. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*. 2006;295:2727-2741.
10. Scher HI, Fizazi K, Saad F, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med*. 2012;367:1187-1197.
13. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005;353:1673-1684.
14. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol*. 2003;21:2787-2799.
15. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
16. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
18. Eklund JW, Kuzel TM. Denileukin diftitox: a concise clinical review. *Expert Rev Anticancer Ther*. 2005;5:33-38.
19. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.

20. Gollob JA. Sorafenib: scientific rationales for single-agent and combination therapy in clear-cell renal cell carcinoma. *Clin Genitourin Cancer*. 2005;4:167-174.
21. Atkins M, Jones CA, Kirkpatrick P. Sunitinib maleate. *Nat Rev Drug Discov*. 2006;5:279-280.
22. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
23. Sekulic A, Migden MR, Oro AE. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *N Engl J Med*. 2012;366:2171-2179.
24. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol*. 2012;12:269-281.
25. Hodi FS, O'Day SJ, McDermott DF. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
26. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
27. Goldman ID, Chattopadhyay Zhao R, Moran R. The antifolates: evolution, new agents in the clinic, and how targeting delivery via specific membrane transporters is driving the development of the next generation of folate analogs. *Current Opin Invest Drugs*. 2010;11:1409-1423.
28. Adjei AA. Pemetrexed (ALIMTA), a novel multitargeted antineoplastic agent. *Clin Cancer Res*. 2004;10:4276-4280.
29. McKendrick J, Coutsouvelis J. Capecitabine: effective oral fluoropyrimidine chemotherapy. *Expert Opin Pharmacother*. 2005;6:1231-1239.
31. Hijiya N, Barry E, Arceci RJ. Clofaribine in pediatric acute leukemia: current findings and issues. *Pediatr Blood Cancer*. 2012;59:417-422.
34. Ratain MJ. From bedside to bench to bedside to clinical practice: an odyssey with irinotecan. *Clin Cancer Res*. 2006;12:1658-1660.
37. Cheson BD, Rummel MJ. Bendamustine: rebirth of an old drug. *J Clin Oncol*. 2009;27:1492-1501.
40. Ueda H, Nakajima H, Hori Y, et al. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. *J Antibiot (Tokyo)*. 1994;47:301-310.
41. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
42. Coffier B, Pro B, Prince HM, et al. Results from a pivotal, open label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol*. 2012;30:631-636.
43. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anti cancer drug. *Nat Biotechnol*. 2007;1:84-90.
46. Desai N, Trieu V, Damascelli B, Soon-Shiong P. SPARC expression correlates with tumor response to albumin-bound paclitaxel in head and neck cancer patients. *Transl Oncol*. 2009;2:59-64.
47. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med*. 2004;351:1513-1520.
50. Wang J, Lou Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs*. 2003;14:13-19.
51. Senter PD, Sievers EL. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat Biotechnol*. 2012;30:631-637.
53. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annu Rev Med*. 2006;57:33-47.
55. Moreau P, Pylypenko H, Grosicki S. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. *Lancet Oncol*. 2011;12:431-440.
56. Hait WN, Jin S, Yang JM. A matter of life or death (or both): understanding autophagy in cancer. *Clin Cancer Res*. 2006;12:1961-1965.

This page intentionally left blank

References

1. Bonnet D, Dick JE. Human myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730-737.
2. Vira D, Basak SK, Veena MS, et al. Cancer stem cells, micro RNAs, and therapeutic strategies including natural products. *Cancer Metastasis Rev*. 2012;31:733-571.
3. Santen RJ, Lipton A, Kendall J. Successful medical adrenalectomy with aminoglutethimide. Role of altered drug metabolism. *JAMA*. 1974;230:1661-1665.
4. de Bono JS, Logothetis CJ, Molina A, et al. Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*. 2011;364:1995-2005.
5. Folkman J. Angiogenesis. *Annu Rev Med*. 2006;57:1-18.
6. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature*. 1993;362:841-844.
7. Presta LG, Chen H, O'Connor SJ, et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res*. 1997;57:4593-4599.
8. Bagnasco L, Piras D, Parodi S, et al. Role of angiogenesis inhibitors in colorectal cancer: sensitive and insensitive tumors. *Curr Cancer Drug Targets*. 2012;12:303-315.
9. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*. 2006;295:2727-2741.
10. Scher HI, Fizazi K, Saad F, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. *N Engl J Med*. 2012;367:1187-1197.
11. Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature*. 1986;319:226-230.
12. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177-182.
13. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med*. 2005;353:1673-1684.
14. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol*. 2003;21:2787-2799.
15. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350:2129-2139.
16. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304:1497-1500.
17. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med*. 2002;346:645-652.
18. Eklund JW, Kuzel TM. Denileukin diftitox: a concise clinical review. *Expert Rev Anticancer Ther*. 2005;5:33-38.
19. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
20. Gollob JA. Sorafenib: scientific rationales for single-agent and combination therapy in clear-cell renal cell carcinoma. *Clin Genitourin Cancer*. 2005;4:167-174.
21. Atkins M, Jones CA, Kirkpatrick P. Sunitinib maleate. *Nat Rev Drug Discov*. 2006;5:279-280.
22. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
23. Sekulic A, Migden MR, Oro AE. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *N Engl J Med*. 2012;366:2171-2179.
24. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol*. 2012;12:269-281.
25. Hodi FS, O'Day SJ, McDermott DF. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
26. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
27. Goldman ID, Chattopadhyay Zhao R, Moran R. The antifolates: evolution, new agents in the clinic, and how targeting delivery via specific membrane transporters is driving the development of the next generation of folate analogs. *Current Opin Invest Drugs*. 2010;11:1409-1423.
28. Adjei AA. Pemetrexed (ALIMTA), a novel multitargeted antineoplastic agent. *Clin Cancer Res*. 2004;10:4276-4280.
29. McKendrick J, Coutsouvelis J. Capecitabine: effective oral fluoropyrimidine chemotherapy. *Expert Opin Pharmacother*. 2005;6:1231-1239.
30. Hertel LW, Boder GB, Kroin JS, et al. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). *Cancer Res*. 1990;50:4417-4422.
31. Hijiya N, Barry E, Arceci RJ. Clofaribine in pediatric acute leukemia: current findings and issues. *Pediatr Blood Cancer*. 2012;59:417-422.
32. Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem*. 1989;58:351-375.
33. Potmesil M. Camptothecins: from bench research to hospital wards. *Cancer Res*. 1994;54:1431-1439.
34. Ratain MJ. From bedside to bench to bedside to clinical practice: an odyssey with irinotecan. *Clin Cancer Res*. 2006;12:1658-1660.
35. Goodman LS, Wintrobe MM, Dameshek W, Goodman MJ, Gilman A, McLennan MT. Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan. *JAMA*. 1984;251:2255-2261.
36. Friedman HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. *Clin Cancer Res*. 2000;6:2585-2597.
37. Cheson BD, Rummel MJ. Bendamustine: rebirth of an old drug. *J Clin Oncol*. 2009;27:1492-1501.
38. Rosenberg B, Vancamp L, Krigas T. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature*. 1965;205:698-699.
39. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. Cellular and molecular pharmacology of oxaliplatin. *Mol Cancer Ther*. 2002;1:227-235.
40. Ueda H, Nakajima H, Hori Y, et al. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. *J Antibiot (Tokyo)*. 1994;47:301-310.
41. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
42. Coffier B, Pro B, Prince HM, et al. Results from a pivotal, open label, phase II study of romidepsin in relapsed or refractory peripheral T-cell lymphoma after prior systemic therapy. *J Clin Oncol*. 2012;30:631-636.
43. Marks PA, Breslow R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anti cancer drug. *Nat Biotechnol*. 2007;1:84-90.
44. Wall ME, Wani MC. Camptothecin and taxol: discovery to clinic. Thirteenth F. Bruce Cain Memorial Award Lecture. *Cancer Res*. 1995;55:753-760.
45. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. *Nature*. 1979;277:665-667.
46. Desai N, Trieu V, Damascelli B, Soon-Shiong P. SPARC expression correlates with tumor response to albumin-bound paclitaxel in head and neck cancer patients. *Transl Oncol*. 2009;2:59-64.
47. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med*. 2004;351:1513-1520.
48. Zhang CC, Yang JM, White E, Murphy M, Levine A, Hait WN. The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. *Oncogene*. 1998;16:1617-1624.
49. Bollag DM, McQueney PA, Zhu J, et al. Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. *Cancer Res*. 1995;55:2325-2333.
50. Wang J, Lou Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs*. 2003;14:13-19.
51. Senter PD, Sievers EL. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat Biotechnol*. 2012;30:631-637.
53. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. *Annu Rev Med*. 2006;57:33-47.
54. Ni H, Ergin M, Huang Q, et al. Analysis of expression of nuclear factor kappa B (NF-kappa B) in multiple myeloma: downregulation of NF-kappa B induces apoptosis. *Br J Haematol*. 2001;115:279-286.
55. Moreau P, Pylypenko H, Grosicki S. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. *Lancet Oncol*. 2011;12:431-440.
56. Hait WN, Jin S, Yang JM. A matter of life or death (or both): understanding autophagy in cancer. *Clin Cancer Res*. 2006;12:1961-1965.

Branimir I. Sikic

47

Natural and Acquired Resistance to Cancer Therapies

Chemotherapy of cancers has resulted in some notable successes, such as the cure of the majority of patients with childhood leukemias, testicular carcinomas, and Hodgkin's and non-Hodgkin lymphomas. In other cancer types, such as breast, colorectal, and lung, chemotherapy also cures some patients when used in the adjuvant setting, that is, after debulking of the cancers with surgery and/or radiotherapy. However, for these common epithelial cancers, cure is rarely attained when the cancers have reached the stage of known metastatic disease. In such cases of advanced-stage cancers, chemotherapies may provide clinical benefit in the form of temporary remission of tumors and abatement of symptoms. In most patients, resistance to therapies is manifested as growth of cancers despite administration of drugs or in relapse or regrowth after a remission. In this chapter, we review reasons for failure of systemic cancer therapies (Table 47-1).

Pharmacologic and Physiologic Causes of Treatment Failure

Inadequate Drug Dose

In addition to cellular mechanisms of drug resistance, pharmacologic and physiologic factors may play a role in outcomes of cancer therapy. Pharmacologic factors include inadequate drug dosing or suboptimal scheduling of agents.[1-3] Traditionally, cytotoxic drugs have been administered at the maximum tolerated doses based on toxicities to normal tissues. Increased therapeutic efficacies with increasing doses, up to maximum levels of acceptable toxicity, have been demonstrated for virtually all cytotoxins in preclinical models and are supported by clinical data relating dose to efficacy in many cancers.[3]

For recently developed targeted drugs, such as kinase inhibitors, the optimal dose may not be the maximum tolerated dose in patients. The relationships among antitumor efficacy, side effects, pharmacodynamic endpoints, and dose of these agents are complex and require novel methodologies and study designs.

Suboptimal Schedule of Drug Administration

With regard to schedule, drugs with short half-lives and cell cycle phase–specific mechanisms of action are typically more active with continuous drug exposure schedules, such as repeated dosing and continuous infusions.[1] Examples include the antimetabolites cytarabine and 5-fluorouracil, the antibiotic bleomycin, and topoisomerase I inhibitors.

Drug Sanctuary Sites (Central Nervous System and Testis)

A physiologic cause for drug resistance is inadequate distribution of drugs to the central nervous system (CNS) and testis, because of the blood-brain and blood-testicular barriers. A major component of these barriers is endothelial cell expression of the multidrug transporter P-glycoprotein.[4,5] Thus, relapse in the CNS and testis became evident as major sites of treatment failure after most children with acute lymphoblastic leukemia achieved a clinical complete remission. New treatment strategies such as high-dose methotrexate and intrathecal administration of chemotherapy were devised to circumvent the problem of relapse in these so-called sanctuary sites.

Poor Drug Diffusion into Cancer Tissues

The physiology of abnormal new blood vessel formation (angiogenesis) and areas of poor blood supply may also limit the ability of anticancer drugs to distribute into cancer tissues and thus result in treatment failure.[6,7] Inhibitors of

tumor angiogenesis such as sunitinib and bevacizumab have been shown to normalize tumor vasculature, thus increasing perfusion and distribution of chemotherapeutic drugs and potentially enhancing therapeutic efficacy.[8]

Cellular Mechanisms of Drug Resistance

Intrinsic versus Acquired Resistance

The various types of cellular mechanisms of drug resistance are depicted in Figure 47-1. These mechanisms can be either intrinsic (i.e., constitutively expressed in the tumor tissue from the outset) or acquired (i.e., derived by mutation or induction of gene expression within the tumor cell population), often after exposure to therapies.[9,10] Intrinsic resistance generally refers to the preexisting expression of cellular defense mechanisms, which are also present in the normal tissues from which the cancer is derived.

Table 47-1 Causes for Failure of Systemic Cancer Therapies

A. Pharmacologic and physiologic mechanisms
1. Inadequate drug dosing
2. Suboptimal schedule of drug administration
3. Sanctuary sites (blood-brain and blood-testicular barriers)
4. Poor diffusion and distribution into tumor tissues
B. Cellular mechanisms of drug resistance
1. Drug efflux transporters
2. Impaired drug uptake
3. Mutation or altered expression of molecular targets
4. Intracellular redistribution of drug
5. Detoxification of drug or intermediate drug product
6. Enhanced DNA repair
7. Decreased drug activation
8. Altered pathways for programmed cell death (apoptosis)

Epithelial tissues such as the colon, kidney, and liver express many transporters and detoxifying enzymes for xenobiotics. These defense mechanisms are also usually expressed in carcinomas originated from those organs and confer resistance to many chemotherapeutic drugs. Normal hematolymphoid tissues are less well defended against xenobiotics, with the exception of certain subtypes (natural killer cells, hematopoietic stem cells).[11] Thus, lymphomas and leukemias express fewer resistance mechanisms and are generally more sensitive to chemotherapies than are epithelial cancers.

The distinction between intrinsic and acquired resistance is blurred in the case of oncogenic mechanisms, such as mutations of *p53* and translocations resulting in high activity of the anti-apoptotic protein BCL-2. These oncogenic mutations result in the inhibition of normal apoptotic mechanisms and thus have a dual effect of causing cancer and resistance to anticancer therapies, which require apoptotic c signaling to kill cancer cells.[12-25] The tissue microenvironment of cancers, involving cell-cell and cell-matrix interactions, may also lead to resistance to cancer therapies via pathways that inhibit apoptosis.[26]

Genetics of Drug Resistance

One of the hallmarks of cancer is genetic instability, resulting in a variety of genetic aberrations, including

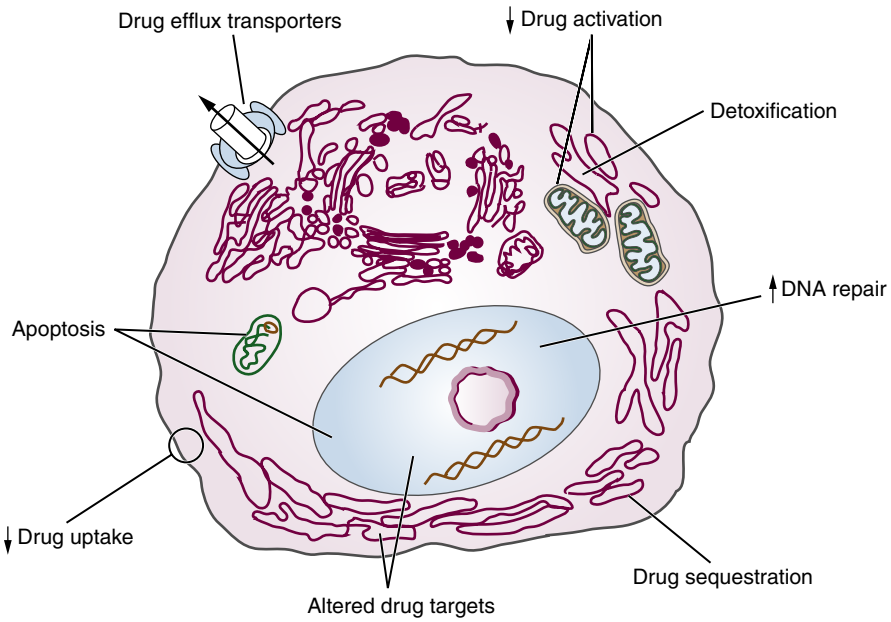


Figure 47-1 Mechanisms of cellular drug resistance.

aneuploidy, point mutations, deletions, gene amplifications, and chromosomal translocations.[27] These result in marked clonal heterogeneity within cancers, resulting in altered gene and protein expression and substantial diversity among cellular populations in primary cancers, metastases, and relapsing tumors after therapies.[28,29] Goldie and Coldman formulated a mathematical model relating rates of generation of drug-resistant mutations to the number of cells in a cancer at diagnosis, the probabilities for cure, and implications for various treatment strategies.[30-32] Studies of human cancer cell lines using Luria-Delbrück fluctuation analysis have demonstrated mutation rates for drug resistance at a frequency of 10^{-6} to 10^{-7} per cell generation.[33-36] Moreover, strategies that suppress one mechanism of resistance (activation of the *MDR1* or *ABCB1* gene) reduce the frequency of acquired resistance and result in the selection of alternative resistance mechanisms.[35]

It is likely that the rate of development of drug-resistant mutants in a cancer varies depending on the nature of the genetic instability of that cancer, the drug mechanism, and the treatment dose and schedule (selection pressure).[30-32,37,38] In general, the concept of selection for acquired resistance implies that mutations either may preexist as small subpopulations within the cancer or may arise during the course of therapy and eventually manifest themselves as regrowth of tumor. The tumor cell population in newly diagnosed metastatic cancers almost always exceeds 1 billion cells (equivalent to 1 g or cubic centimeter of tumor). Thus, it is very likely that cancers that are intrinsically sensitive to any therapeutic agent will also contain one or more drug-resistant clones. This provides a powerful rationale for both combination drug therapies (to lessen the likelihood of doubly resistant clones) and adjuvant therapies of cancers (to cure patients with micrometastatic disease and lower tumor burdens).

Recent genetic studies have provided new insights into clonal populations and the evolution of resistant variants in clinical cancers.[39-41] These studies used DNA sequencing and sampling of multiple tumor sites of primary and metastatic cancers to directly demonstrate heterogeneity within tumors and changes in the distribution of clonal populations after therapies.

Gene amplification, or increase in gene copy number, was first described for the *DHFR* gene as a mechanism for acquired resistance to methotrexate.[42] Amplification of genes is now known to be a prominent feature of the genomic instability of cells and to be a key genetic mechanism involved in oncogenesis (*MYC*, *HER2*, *EGFR*), as well as in drug resistance. It is one of the major mechanisms for increasing the expression of drug-resistance genes, including *MDR1/ABCB1*.[27,43]

Epigenetics and Drug Resistance

In addition to selection of resistant mutants, acquired resistance may develop via epigenetic changes, by induction of resistance gene expression.[44] For example, various cellular stresses, including exposure to ionizing radiation and chemotherapies, have been shown to increase expression of the multidrug transporter gene *MDR1/ABCB1*.[27,45-47] These epigenetic mechanisms such as DNA methylation and histone modifications can contribute to heterogeneity in gene expression and also offer the possibility of reversing drug resistance with drugs such as vorinostat and decitabine that target the epigenome.[48-55] MicroRNAs are a target for epigenetic regulation that can alter drug resistance. Thus, downregulation of the miR-200 family results in epithelial-to-mesenchymal transition (EMT) and upregulation of tubulin beta-3 (TUBB3), which can confer resistance to taxane drugs.[56]

Cancer stem cells (CSCs) represent a subset of cells within a cancer that have the capacity for sustained proliferation and that are thought to be primarily responsible for the growth of cancer. CSCs typically have an EMT phenotype and upregulation of many survival mechanisms, including drug transporters and resistance to apoptosis.[57-60]

Tumor Stroma, Cell-to-Cell Interactions, and Drug Resistance

The tumor microenvironment and, in particular, interactions of stromal cells with cancer cells have been shown to enhance drug resistance.[61-64] The underlying mechanism for this effect is protection from cell death or apoptosis, mediated by both cellular and noncellular components of the tumor microenvironment. These stromal components include cellular adhesion molecules in the extracellular matrix, chemokines such as CSCL12, and integrins. Cross talk between cancer-associated fibroblasts and malignant cells in tumors promotes tumor progression and cell survival in part via cell adhesion to fibronectin.[64]

Drug Efflux Transporters

There are approximately 50 ABC transporters (ATP-binding cassette membrane proteins) in the human genome.[65-67] Defective forms of several of these transporters are causes of human genetic diseases, such as cystic fibrosis and Dubin-Johnson syndrome.[65] Several members of the ABC transporter family have the capacity to efflux small molecules, including anticancer drugs, and thus contribute to drug resistance. The major drug-resistance ABC transporter genes

include *MDR1/ABCB1*, several members of the *MRP/ABCC* subgroup, and *ABCG2*.[65,67,68]

P-glycoprotein (P-gp), the product of the *MDR1/ABCB1* gene, is the most prevalent ABC drug-resistance transporter and has been extensively studied.[69-73] The protein has a molecular mass of 180 kDa, with 12 transmembrane segments and two intracytoplasmic ATP binding domains (Figure 47-2). High-resolution electron microscopy has revealed that the transmembrane segments form a pore, and drug binding sites have been identified within this pore. Access of drugs to the transporter is thought to occur both via the cytoplasm and by diffusion within the membrane. P-gp is a transporter with very broad substrate specificity, including approximately a third of all anticancer drugs, as well as many other drugs used in other areas of medicine. Active efflux of drugs is mediated by conversion of ATP to ADP. Theories regarding the molecular mechanism of drug extrusion include an ATPase-mediated conformational change in the protein producing a "flippase" action, which exposes substrate drugs to the extracellular environment, and a "membrane vacuum cleaner" function in which drugs access the transporter via the bilipid plasma membrane.[70] The direct role of P-gp in conferring multidrug resistance has been confirmed by transfection of the gene in cellular models.[72]

P-gp is expressed in many normal tissues, where it serves as a barrier to drug absorption (small bowel and colon), a barrier to tissue entry (endothelial cells of the CNS, testis, and placenta), and to facilitate drug excretion (biliary tract of the liver and proximal tubule of the kidney.[4,73] It is also highly expressed in cancers derived from these tissues (colorectal, renal) and is one of the constitutive mechanisms of drug resistance in these cancers.

P-gp expression in cancers results in a classical multidrug-resistance phenotype, with high degrees of resistance to the drugs that are transport substrates for the protein. These drug substrates include the anthracyclines (doxorubicin, daunorubicin, idarubicin, and epirubicin), vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), mitoxantrone, and dactinomycin.[70] Many

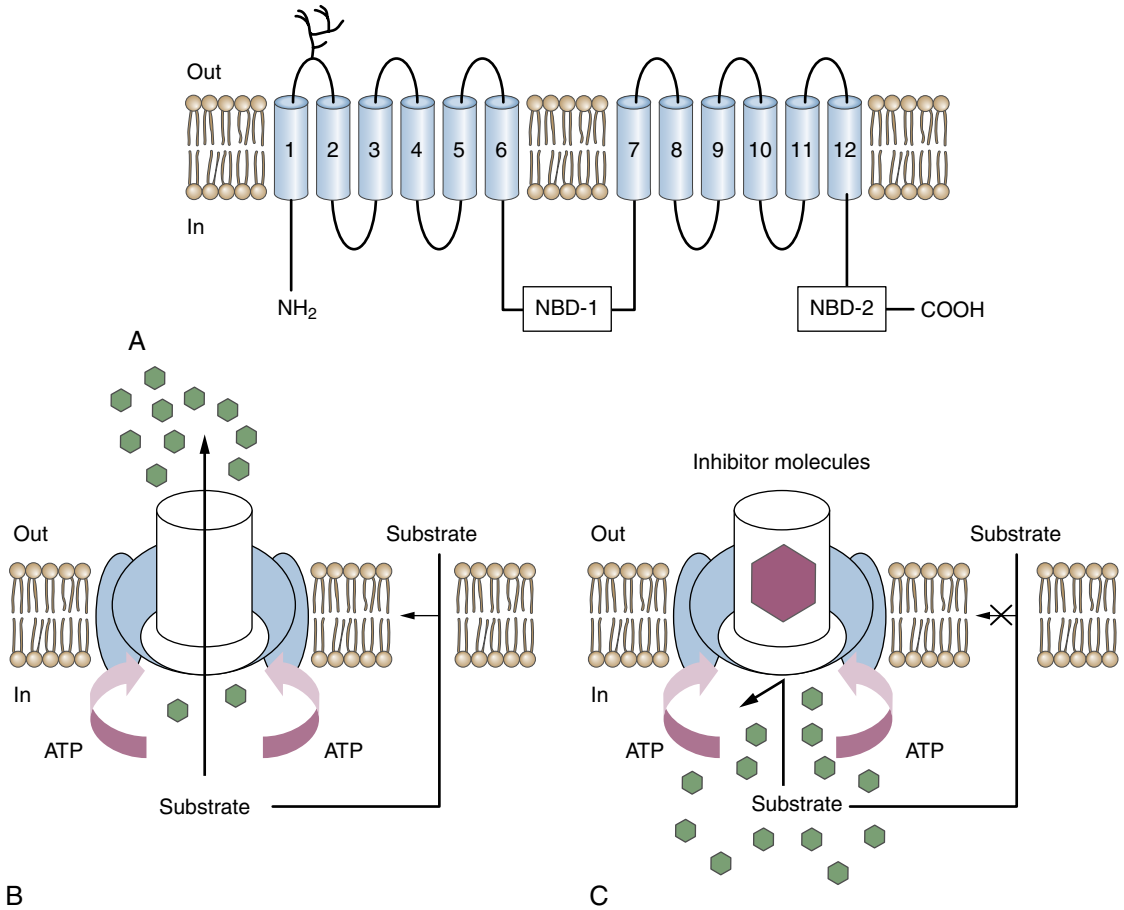


Figure 47-2 Structure and mechanism of action of P-glycoprotein (P-gp). **(A)** Diagram of P-gp showing the 12 transmembrane segments, two nucleotide binding domains (NBDs), and extracellular glycosylation. **(B)** P-gp forms a central pore and requires ATPase activity to pump drugs out of the cell. **(C)** Inhibitors of P-gp function prevent drug efflux, resulting in increased intracellular drug accumulation and enhanced killing of multidrug-resistant cells.

newer, targeted drugs such as imatinib are also transport substrates for P-gp.[74]

The clinical significance of P-gp in drug resistance is supported by evidence that its expression confers an adverse prognosis in many tumor types, including acute myeloid leukemias (AMLs), acute lymphoid leukemias, lymphomas, myeloma, breast and ovarian cancers, and sarcomas.[9,75-81] In AML, P-gp is expressed in more than 70% of specimens from patients older than age 60, versus 30% to 40% of patients up to age 60, and its expression correlates with reduced rates of complete remission and shorter survival.[80] In breast cancers, P-gp expression occurs in 40% to 50% of specimens and is associated with decreased rates of remission to P-gp substrate drugs (taxanes and anthracyclines).[81] Selection of multidrug-resistant (MDR) subclones within cancer populations is suggested by evidence that P-gp expression is more frequent in leukemias and breast cancers after patients have relapsed from prior therapy with MDR-related chemotherapy drugs.[80,81]

The prevalence and adverse prognostic effects of P-gp in many cancers have led to attempts to reverse MDR by combining chemotherapy with inhibitors of P-gp.[80,82-84] These clinical trials to reverse or modulate MDR have used a variety of competitive and noncompetitive inhibitors of P-gp, including verapamil, cyclosporine, quinine, the cyclosporine analog valspodar, and others. In general, these attempts have not resulted in proven clinical benefit. The reasons for these failures are multiple and include the following: inadequate concentrations of MDR-reversing agents because of toxicities to normal tissues, lack of specificity of P-gp inhibition leading to drug interactions and off-target effects, use of an unselected patient population including patients who did not express P-gp, and coexpression of other mechanisms of drug resistance.[80,82-84] A particularly problematic issue is the co-inhibition by cyclosporins and other MDR inhibitors of other ABC transporters as well as the mixed-function oxidase CYP 3A4, resulting in the need to reduce doses of chemotherapeutic drugs while attempting to sensitize P-gp–expressing cancer cells.[84-87] Despite these issues, cyclosporine has been shown to moderately increase complete remission rates and to significantly prolong survival in a randomized clinical trial in AML.[80,88] A more potent and specific inhibitor of P-gp, zosuquidar, has not prolonged survival in AML, although the schedule of administration of the drug in this trial was suboptimal.[89]

Several members of the *MRP* or ABCC gene family also function as drug transporters.[68,90-97] The MDR-associated protein (the *MRP1/ABCC1* gene) confers resistance to anthracyclines, vinca alkaloids, and epipodophyllotoxins and preferentially transports glutathione conjugates of substrate drugs.[90,91,94-97] In general, *MRP1* is not as strongly associated with clinical drug resistance and prognosis as *MDR1*, and clinical strategies for reversing resistance related to *MRP1* have not been developed. The *MRP2/ABCC2* gene encodes the canalicular multiple organic anion transporter, which is expressed at high levels in the biliary tract, and transports glucuronide and glutathione conjugates of drugs, including anthracyclines. It plays a role in hepatic excretion of anticancer drugs, but its role in drug resistance is not clear.[92,95] Its hereditary deficiency results in the Dubin-Johnson syndrome.[68] The transporter encoded by the *MRP3/ABCC3* gene confers low-level resistance to epipodophyllotoxins as well as to methotrexate.[68,93] *MRP4/ABCC4* and *MRP5/ABCC5* confer resistance to anionic purines and other nucleotide analogs and their metabolites.[68,98]

ABCG2 (BCRP) is another member of the ABC family, implicated in clinical resistance to the anthracenedione drug mitoxantrone and the camptothecins.[99,100] This transporter is 72 kDa in size, less than half the size of the ABCB and ABCC subgroups, and is thought to require dimerization for its function. It is variably expressed in AML and is a negative prognostic factor in that disease.[101-103] Together with P-gp, *ABCG2* is constitutively expressed in both normal hematopoietic and leukemic stem cells[104,105] and is a marker of cancer stem cells.[60]

Polymorphisms in the DNA sequence of *ABCB1* and other ABC transporters are being studied for their relationship to drug disposition, efficacy, and toxicities.[66] Single-nucleotide polymorphisms of the *ABCB1* gene (C1236T, G2677T, and C3435T), which have been associated with altered drug absorption or disposition in some studies, were not found to effect complete remission and survival in patients with AML.[106] The function and clinical significance of the ABC transporter family in anticancer drug resistance continue to be investigated.

Two membrane proteins involved in the efflux of copper, ATP7A and ATP7B, have been shown to also transport the platinum drugs and contribute to resistance to cisplatin, carboplatin, and oxaliplatin.[107,108]

Impaired Drug Uptake

Cellular entry of most anticancer agents is via passive diffusion. However, some drugs are also transported into cells by membrane proteins, and the expression and activity of these proteins are determinants of cellular sensitivity or resistance. Methotrexate enters cells by means of the reduced folate carrier, and decreased expression of this protein results in relative resistance to the drug.[109] Reduced drug uptake has also been observed in some cells resistant to platinum drugs.[110] The major copper influx transporter, CTR1, been implicated in the regulation of intracellular accumulation of cisplatin, carboplatin, and oxaliplatin.[107]

Mutation or Altered Expression of Molecular Targets

As previously mentioned, the first description of gene amplification as a genetic phenomenon and as a mechanism for acquired drug resistance was the discovery of amplified dihydrofolate reductase *(DHFR)* genes in a cell line selected by exposure to increasing concentrations of methotrexate.[42] Multiple copies of *DHFR* were identified in extrachromosomal fragments of DNA, termed *double minute chromosomes* (DMs), in the methotrexate-resistant cells. Resistance in these cells was unstable because DMs were not normally replicated in the absence of drug selection.[111] Subsequently, other methotrexate-resistant cells were found to have multiple gene copies of *DHFR* integrated into the genome, in areas of "homogeneously staining regions," or HSRs. HSRs are more stable because they are integrated into the genome and included in the normal process of DNA replication.

Several important classes of anticancer drugs (vincas, taxanes, epothilones) act by binding to β tubulins and altering the dynamic instability of microtubules (Figure 47-3).[112] Alterations in β tubulins, including mutations and changes in the proportion of β-tubulin isoforms, particularly the class III isoform, have been implicated in resistance to taxanes.[112-115] Vinca alkaloids inhibit tubulin polymerization and thus have opposing effects to those of taxanes and epothilones, which stabilize polymerized microtubules. These opposing mechanisms of action may be reflected in reciprocal effects of changes in tubulin content or isotype expression on vinca and taxane sensitivities, with resistance to one class of drugs accompanied by increased sensitivity to the other.[112]

Although mutations in β-tubulin that alter taxane binding have been found to confer resistance in cellular models, such mutations have not been found in various human cancer clinical specimens.[116,117]

The microtubule binding protein, MAP-Tau, binds to a site on β-tubulin overlapping with taxanes and affects microtubule dynamic instability. Its expression has been associated with resistance to the taxane drug paclitaxel in breast cancer specimens.[118,119] Other mechanisms of resistance to antitubulin drugs include the P-gp transporter (for taxanes and vincas),[81] the cell spindle checkpoint control pathway,[120] and regulation of programmed cell death or apoptosis.[112,121]

The epothilones are a new class of antitubulin cytotoxic drugs whose binding site on tubulins overlaps with the taxanes.[122] In contrast to taxanes, epothilones are not transport substrates for P-gp and therefore have potential antitumor efficacy in cancers that are multidrug resistant because of P-gp expression.[122,123] However, they are likely to share some of the target-related mechanisms of resistance to taxanes, such as factors that affect microtubule dynamicity and regulation of apoptosis.

Topoisomerase I and II are drug targets for camptothecin and epipodophyllotoxin drugs, respectively, and mutations or altered expression of these enzymes have been shown to cause cellular resistance to these drugs.[33,124-129] Because drug-induced DNA breakage is proportional to the amount of topoisomerase II enzyme, decreased enzyme content is associated with resistance, and higher enzyme content with drug sensitivity.[33,125,128]

Alteration of drug targets is an important mechanism of resistance for new, targeted drugs, such as the tyrosine

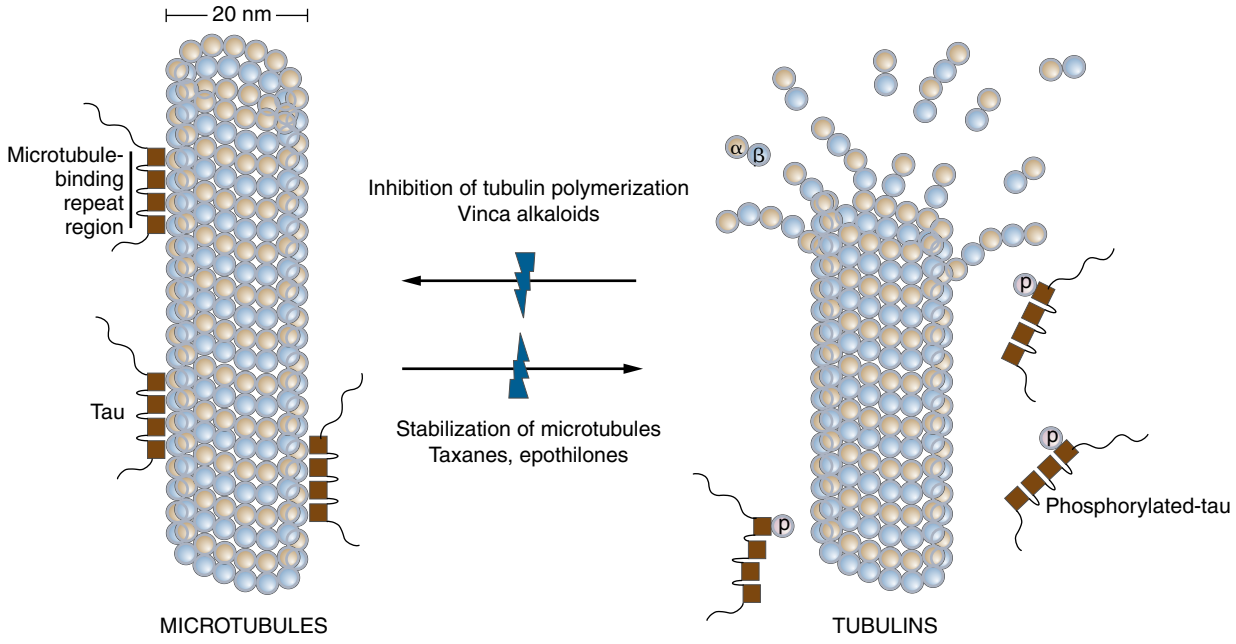


Figure 47-3 Mechanism of action of tubulin polymerizing and microtubule-stabilizing drugs.

kinase inhibitors (Figure 47-4).[74,130] For the drug imatinib, an inhibitor of the fusion oncoprotein gene *BCR/ABL*, point mutations in the kinase domain of its target are a major mechanism of acquired resistance in chronic myeloid leukemias (CMLs).[131] More than 30 such mutations that confer resistance to imatinib have been identified. Because resistance to imatinib occurs at a rate of around 3% of patients per year of drug therapy, such mutations occur relatively infrequently. The drug dasatinib, a potent inhibitor of the *BCR/ABL* kinase, has been shown to inhibit almost all of these mutant kinases and to produce remissions in imatinib-resistant CML.[131] One *BCR/ABL* mutant, T351I, remains resistant to both imatinib and dasatinib, although other new drugs are being developed for this double-resistant mutation. BCR/ABL gene amplification can also result in resistance to the kinase inhibitors in CML.[132]

Intracellular Redistribution of Drug

Intracellular drug sequestration of anthracyclines has been observed in cellular models with high expression of the major vault protein (MVP), also known as *LRP*.[133] Vaults are barrel-like cytoplasmic organelles with a molecular mass of 13 MDa, which are thought to function in intracellular transport. In addition to high expression of MVP in some cellular models of drug resistance, this protein is variably expressed in acute myeloid leukemias and may be a factor in clinical drug resistance in that disease.[134]

Detoxification of Drug or Intermediate Drug Product

Metabolic inactivation of drugs is a mechanism of resistance to many agents. Thus, cytidine deaminase activity can result in resistance to cytarabine.[135] Dihydropyrimidine dehydrogenase catabolism of 5-fluorouracil is a determinant of activity of that agent.[136]

The DNA-binding glycopeptide drug bleomycin is inactivated by an aminopeptidase termed *bleomycin hydrolase*.[137] Most cancers are resistant to bleomycin and have high levels of this enzyme, whereas sensitive tumors (germ cell cancers, lymphomas, squamous carcinomas) have low levels. Similarly, most normal tissues have high levels of bleomycin hydrolase, but the two major sites of toxicity, lung and skin, express low levels.[137]

For electrophilic DNA alkylating agents and platinum drugs, detoxification via nucleophilic sulfur-containing compounds is an important class of resistance pathways.[108] Glutathione reductases are an important class of detoxifying enzymes that can generate resistance to such drugs by conjugation with glutathione.[138-148] Moreover, as previously noted, some members of the MRP family of transporters can efflux glutathione conjugates of cytotoxic drugs, so that

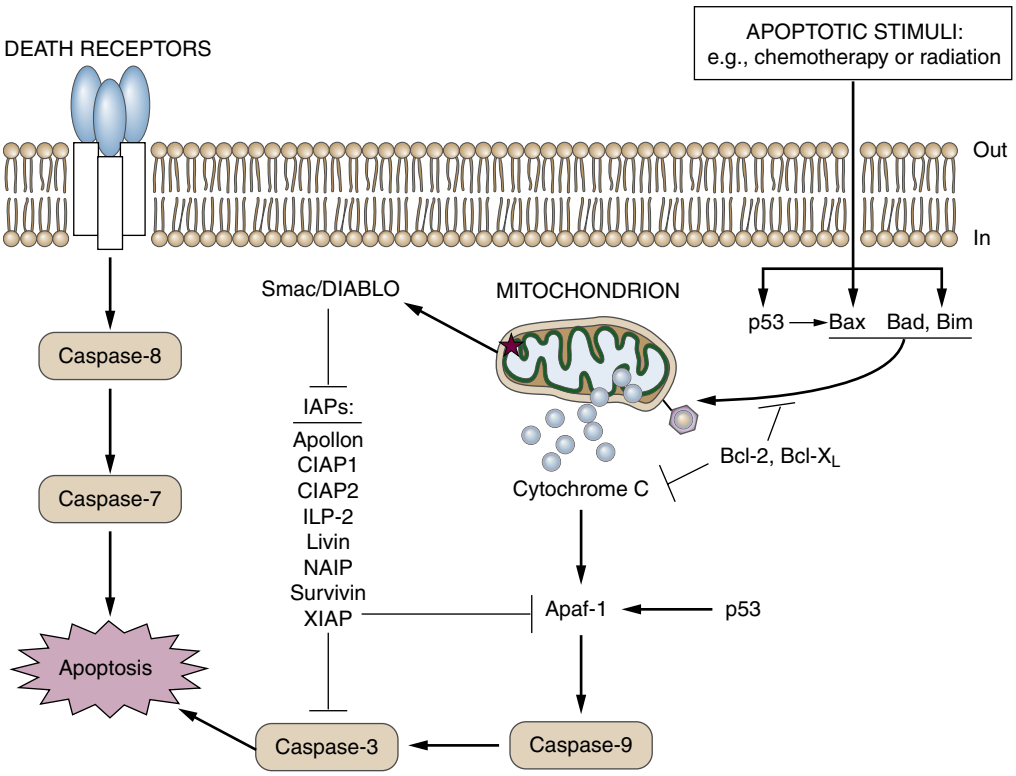


Figure 47-4 Cellular pathways of programmed cell death, or apoptosis.

metabolic detoxification is coupled to outward transport of toxins.[68,95,97]

Enhanced DNA Repair

DNA repair pathways are important determinants of response to alkylating agents and platinum drugs.[108,149-152] Nucleotide excision repair (NER) is a complex, highly regulated process involving more than 30 proteins. Moreover, two general pathways are involved: global genomic NER, which repairs damage in transcriptionally silent areas, and transcription-coupled NER, which repairs damage to the actively transcribed DNA strand. The steps in NER include recognition of the damaged DNA, DNA unwinding, incision, degradation, polymerization, and ligation.[151] Evidence for the role of many DNA repair genes in response to both DNA-damaging drug and ionizing radiation derives in part from studies of genetic defects such as ataxia telangiectasia, xeroderma pigmentosum, and Bloom syndrome, in which hypersensitivity to DNA-damaging agents has been observed.

Among the many genes involved in NER, recent attention has focused on *ERCC1*. High expression of the DNA excision repair gene *ERCC1*, which is involved in repair of DNA adducts from alkylating agents and platinum drugs, has been shown to correlate with adverse outcomes in patients with advanced-stage non–small-cell lung cancers treated with cisplatin-based chemotherapy.[153] In earlier stages of lung cancer, patients whose tumors did not express ERCC1 benefited significantly from cisplatin adjuvant chemotherapy, whereas patients whose tumors expressed *ERCC1* did not benefit from the chemotherapy.[154] Paradoxically, high expression of ERCC1 was found to be a favorable prognostic factor for survival in patients with early stages of lung cancer, in the absence of adjuvant chemotherapy.[154-156]

O6-Methylguanyl-methyl-transferase (MGMT) is particularly important in resistance to the nitrosourea carmustine and the DNA-methylating agent temozolomide.[149,151] MGMT has been identified as a key factor in clinical outcomes in brain tumors, and drugs to deplete MGMT are being developed as potential therapeutic approaches to modulate drug resistance.[149]

Decreased Drug Activation

Most antimetabolite drugs generally require metabolic activation to generate their active nucleoside or nucleotide moiety, via kinases and phosphoribosyl transferases.[157] Thus, for cytarabine, generation of ara-dCTP levels intracellularly is an important determinant of antitumor efficacy.[157] In the case of 5-fluorouracil, activation of the drug requires formation of 5-fluorodeoxyuridine monophosphate (FdUMP).[157] In addition, optimal inhibition of thymidylate synthase by 5-fluorouracil depends in part on intracellular levels of the cofactor 5,10-methylene tetrahydrofolate.[158]

The oxazaphosphorine mustards (cyclophosphamide and ifosfamide) are prodrugs that are activated predominantly in liver tissue by mixed function oxidases (CYP enzymes).[150] Although the major mechanisms of resistance to these drugs are thought to be inactivation of alkylating metabolites by thiol compounds, as well as DNA repair mechanisms, variable levels of mixed function oxidase activity within cancers may also be a determinant of their activity.

Altered Pathways for Programmed Cell Death (Apoptosis)

Pathways for the regulation of programmed cell death or apoptosis are important both in oncogenesis and as determinants of response to cancer therapies (Figure 47-5).[12-25] *BCL2* is oncogenic in many B-cell lymphomas, where its expression is upregulated by chromosomal translocations and other mechanisms. It also functions to protect cells from apoptosis after radiation, glucocorticoids, and chemotherapies.[13,14,25] The *BCLX* gene has long and short forms, encoding the proteins bcl-xL and bcl-xS, which serve to inhibit and promote apoptosis, respectively.[24] Both the BCL-2 and the inhibitor of apoptosis (IAP or BIRC) families of regulators of cell death are currently being explored as targets to sensitize drug-resistant cancer cells to chemotherapies.[159,160]

The relationship between oncogenesis and drug sensitivity or resistance is also exemplified by the p53 pathway, which is mutated in the majority of human cancers. Normal p53 function is essential for the efficient functioning of the mitochondrially mediated apoptotic pathway, particularly in response to DNA-damaging agents, including ionizing radiation and many chemotherapeutic drugs, such as alkylating agents, platinums, anthracyclines, and topoisomerase inhibitors.[12,15,22,23]

Individualization of Therapy Based on Predictive Multigenic Markers

Knowledge about mechanisms of drug resistance, molecular targets of drugs, and signaling pathways related to treatments is enabling more precise predictive molecular testing of drug efficacy.[10] Historically, such approaches were pioneered in the treatment of breast cancer by the use of hormone receptor measurements to guide hormonal therapy

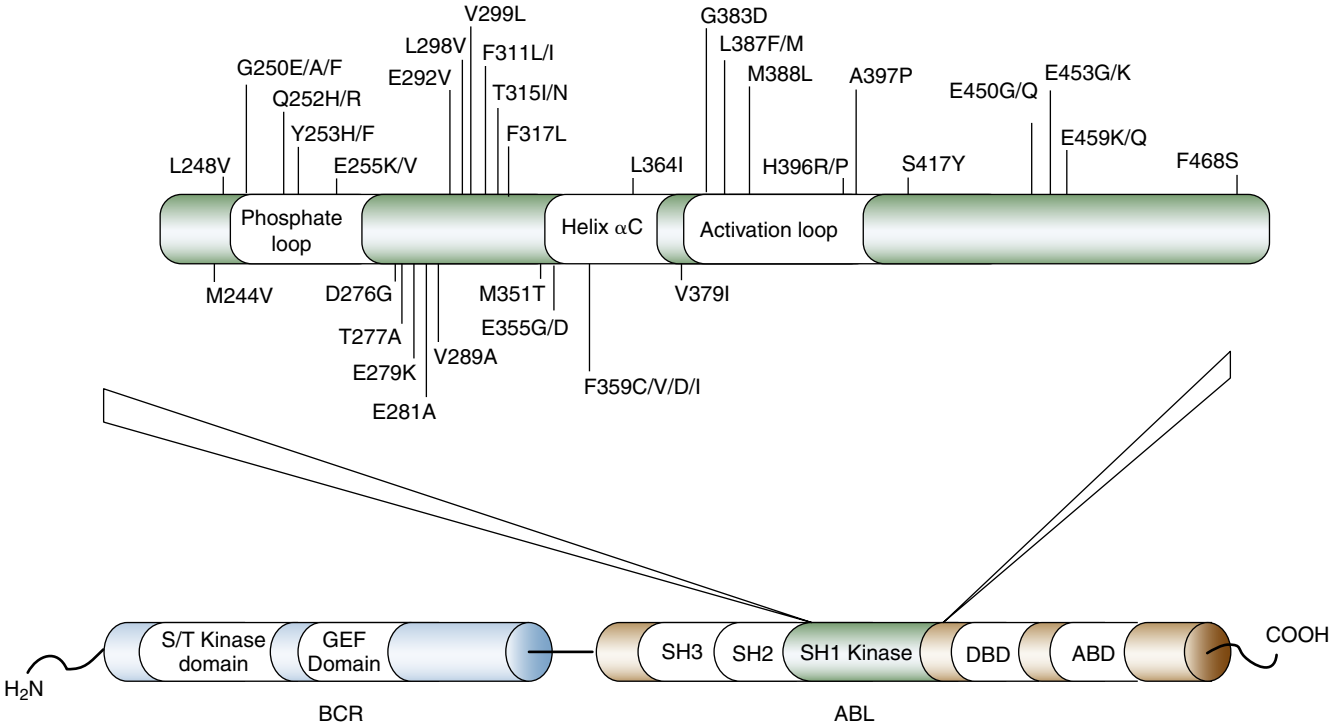


Figure 47-5 Structure of the fusion oncoprotein BCR/ABL, depicting the point mutations that result in resistance to imatinib in chronic myeloid leukemias.

and testing for overexpression or amplification of the *HER2* gene to identify breast cancer patients for trastuzumab therapy. The ability to determine genome-wide expression by microarray analysis has resulted in the identification of candidate gene profiles that are associated with remissions to drugs or drug combinations.[161,162] Such approaches may lead to increasing individualization of therapy with the use of genomic or proteomic panels of predictive markers, but prospective validation of such markers in clinical trials has been difficult.

Key References

3. Marangolo M, Bengala C, Conte PF, et al. Dose and outcome: the hurdle of neutropenia (Review). *Oncol Rep*. 2006;16:233-248.
6. Kyle AH, Huxham LA, Yeoman DM, et al. Limited tissue penetration of taxanes: a mechanism for resistance in solid tumors. *Clin Cancer Res*. 2007;13:2804-2810.
7. Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer*. 2006;6:583-592.
8. Matsumoto S, Batra S, Saito K, et al. Antiangiogenic agent sunitinib transiently increases tumor oxygenation and suppresses cycling hypoxia. *Cancer Res*. 2011;71:6350-6359.
10. Quintieri L, Fantin M, Vizler C. Identification of molecular determinants of tumor sensitivity and resistance to anticancer drugs. *Adv Exp Med Biol*. 2007;593:95-104.
11. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5:275-284.
21. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006;25:4798-4811.
26. Morin PJ. Drug resistance and the microenvironment: nature and nurture. *Drug Resist Updat*. 2003;6:169-172.
27. Fojo T. Multiple paths to a drug resistance phenotype: mutations, translocations, deletions and amplification of coding genes or promoter regions, epigenetic changes and microRNAs. *Drug Resist Updat*. 2007;10:59-67.
29. Greaves M, Maley CC. Clonal evolution in cancer. *Nature*. 2012;481:306-313.
38. Chen KG, Wang YC, Schaner ME, et al. Genetic and epigenetic modeling of the origins of multidrug-resistant cells in a human sarcoma cell line. *Cancer Res*. 2005;65:9388-9397.
39. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
40. Lee AJ, Swanton C. Tumour heterogeneity and drug resistance: personalising cancer medicine through functional genomics. *Biochem Pharmacol*. 2012;83:1013-1020.
41. Swanton C. Intratumor heterogeneity: evolution through space and time. *Cancer Res*. 2012;72:4875-4882.
43. Wang YC, Juric D, Francisco B, et al. Regional activation of chromosomal arm 7q with and without gene amplification in taxane-selected human ovarian cancer cell lines. *Genes Chromosomes Cancer*. 2006;45:365-374.
44. Matei D, Fang F, Shen C, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res*. 2012;72:2197-2205.
48. Wilting RH, Dannenberg JH. Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. *Drug Resist Updat*. 2012;15:21-38.
49. Balch C, Nephew KP. Epigenetic targeting therapies to overcome chemotherapy resistance. *Adv Exp Med Biol*. 2013;754:285-311.
50. Zeller C, Dai W, Steele NL, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. *Oncogene*. 2012;31:4567-4576.
51. Shen DW, Pouliot LM, Hall MD, et al. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. *Pharmacol Rev*. 2012;64:706-721.
52. Bhatla T, Wang J, Morrison DJ, et al. Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. *Blood*. 2012;119:5201-5210.

53. Chen KG, Sikic BI. Molecular pathways: regulation and therapeutic implications of multidrug resistance. *Clin Cancer Res*. 2012;18:1863-1869.
54. Rosenzweig SA. Acquired resistance to drugs targeting receptor tyrosine kinases. *Biochem Pharmacol*. 2012;83:1041-1048.
55. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
56. Mongroo PS, Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol Ther*. 2010;10:219-222.
57. Basile KJ, Aplin AE. Resistance to chemotherapy: short-term drug tolerance and stem cell-like subpopulations. *Adv Pharmacol*. 2012;65:315-334.
58. Ricci F, Bernasconi S, Perego P, et al. Ovarian carcinoma tumor-initiating cells have a mesenchymal phenotype. *Cell Cycle*. 2012;11:1966-1976.
59. Smalley M, Piggott L, Clarkson R. Breast cancer stem cells: Obstacles to therapy. *Cancer Lett*. 2012 Apr 30 [Epub ahead of print].
60. Maugeri-Sacca M, Vigneri P, De Maria R. Cancer stem cells and chemosensitivity. *Clin Cancer Res*. 2011;17:4942-4947.
61. Sebens S, Schafer H. The tumor stroma as mediator of drug resistance—a potential target to improve cancer therapy? *Curr Pharm Biotechnol*. 2012;13:2259-2272.
62. Sun Y, Nelson PS. Molecular pathways: involving microenvironment damage responses in cancer therapy resistance. *Clin Cancer Res*. 2012;18:4019-4025.
63. Westhoff MA, Fulda S. Adhesion-mediated apoptosis resistance in cancer. *Drug Resist Updat*. 2009;12:127-136.
64. Flach EH, Rebecca VW, Herlyn M, et al. Fibroblasts contribute to melanoma tumor growth and drug resistance. *Mol Pharm*. 2011;8:2039-2049.
66. Huang Y. Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis Rev*. 2007;26:183-201.
67. Glavinas H, Krajcsi P, Cserepes J, et al. The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr Drug Deliv*. 2004;1:27-42.
70. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, et al. P-glycoprotein: from genomics to mechanism. *Oncogene*. 2003;22:7468-7485.
74. Shukla S, Chen ZS, Ambudkar SV. Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance. *Drug Resist Updat*. 2012;15:70-80.
80. Mahadevan D, List AF. Targeting the multidrug resistance-1 transporter in AML: molecular regulation and therapeutic strategies. *Blood*. 2004;104:1940-1951.
81. Clarke R, Leonessa F, Trock B. Multidrug resistance/P-glycoprotein and breast cancer: review and meta-analysis. *Semin Oncol*. 2005;32:S9-S15.
98. Fukuda Y, Schuetz JD. ABC transporters and their role in nucleoside and nucleotide drug resistance. *Biochem Pharmacol*. 2012;83:1073-1083.
101. Benderra Z, Faussat AM, Sayada L, et al. Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. *Clin Cancer Res*. 2004;10:7896-7902.
102. Benderra Z, Faussat AM, Sayada L, et al. MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. *Clin Cancer Res*. 2005;11:7764-7772.
103. Plasschaert SL, Van Der Kolk DM, De Bont ES, et al. Breast cancer resistance protein (BCRP) in acute leukemia. *Leuk Lymphoma*. 2004;45:649-654.
104. Raaijmakers MH, de Grouw EP, Heuver LH, et al. Breast cancer resistance protein in drug resistance of primitive CD34+38- cells in acute myeloid leukemia. *Clin Cancer Res*. 2005;11:2436-2444.
105. Sikic BI. Multidrug resistance and stem cells in acute myeloid leukemia. *Clin Cancer Res*. 2006;12:3231-3232.
106. van der Holt B, Van den Heuvel-Eibrink MM, Van Schaik RH, et al. ABCB1 gene polymorphisms are not associated with treatment outcome in elderly acute myeloid leukemia patients. *Clin Pharmacol Ther*. 2006;80:427-439.
107. Safaei R, Howell SB. Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. *Crit Rev Oncol Hematol*. 2005;53:13-23.
108. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov*. 2005;4:307-320.
113. Orr GA, Verdier-Pinard P, McDaid H, et al. Mechanisms of taxol resistance related to microtubules. *Oncogene*. 2003;22:7280-7295.
114. Séve P, Mackey J, Isaac S, et al. Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. *Mol Cancer Ther*. 2005;4:2001-2007.
115. Yusuf RZ, Duan Z, Lamendola DE, et al. Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. *Curr Cancer Drug Targets*. 2003;3:1-19.
117. Mesquita B, Veiga I, Pereira D, et al. No significant role for beta tubulin mutations and mismatch repair defects in ovarian cancer resistance to paclitaxel/cisplatin. *BMC Cancer*. 2005;5:101-106.
118. Rouzier R, Rajan R, Wagner P, et al. Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer. *Proc Natl Acad Sci U S A*. 2005;102:8315-8320.
119. Wagner P, Wang B, Clark E, et al. Microtubule Associated Protein (MAP)-Tau: a novel mediator of paclitaxel sensitivity in vitro and in vivo. *Cell Cycle*. 2005;4:1149-1152.
120. Sudo T, Nitta M, Saya H, et al. Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Res*. 2004;64:2502-2508.
121. Sugimura M, Sagae S, Ishioka S, et al. Mechanisms of paclitaxel-induced apoptosis in an ovarian cancer cell line and its paclitaxel-resistant clone. *Oncology*. 2004;66:53-61.
122. Goodin S, Kane MP, Rubin EH. Epothilones: mechanism of action and biologic activity. *J Clin Oncol*. 2004;22:2015-2025.
123. Lee JJ, Swain SM. Development of novel chemotherapeutic agents to evade the mechanisms of multidrug resistance (MDR). *Semin Oncol*. 2005;32:S22-S26.
130. Lackner MR, Wilson TR, Settleman J. Mechanisms of acquired resistance to targeted cancer therapies. *Future Oncol*. 2012;8:999-1014.
131. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance and the road to a cure of chronic myeloid leukemia. *Blood*. 2007;110:2242-2249.
132. Diamond JM, Melo JV. Mechanisms of resistance to BCR-ABL kinase inhibitors. *Leuk Lymphoma*. 2011;52(Suppl 1):12-22.
149. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer*. 2004;4:296-307.
150. Zhang J, Tian Q, Chan SY, et al. Insights into oxazaphosphorine resistance and possible approaches to its circumvention. *Drug Resist Updat*. 2005;8:271-297.
153. Ceppi P, Volante M, Novello S, et al. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. *Ann Oncol*. 2006;17:1818-1825.
154. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*. 2006;355:983-991.
155. Zheng Z, Chen T, Li X, et al. DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. *N Engl J Med*. 2007;356:800-808.
156. Gazdar AF. DNA repair and survival in lung cancer—the two faces of Janus. *N Engl J Med*. 2007;356:771-773.
159. Smolewski P, Robak T. Inhibitors of apoptosis proteins (IAPs) as potential molecular targets for therapy of hematological malignancies. *Curr Mol Med*. 2011;11:633-649.
160. Jendrossek V. The intrinsic apoptosis pathways as a target in anticancer therapy. *Curr Pharm Biotechnol*. 2012;13:1426-1438.
161. Gianni L, Zambetti M, Clark K, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. *J Clin Oncol*. 2005;23:7265-7277.
162. Hess KR, Anderson K, Symmans WF, et al. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. *J Clin Oncol*. 2006;24:4236-4244.

References

1. Carlson RW, Sikic BI. Continuous infusion or bolus injection in cancer chemotherapy. *Ann Intern Med*. 1983;99:823-833.
2. Cassidy J. Chemotherapy administration: doses, infusions and choice of schedule. *Ann Oncol*. 1994;5(suppl 4):25-29 discussion 29–30.
3. Marangolo M, Bengala C, Conte PF, et al. Dose and outcome: the hurdle of neutropenia (Review). *Oncol Rep*. 2006;16:233-248.
4. Cordon-Cardo C, O'Brien JP, Casals D, et al. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci U S A*. 1989;86:695-698.
5. Schinkel AH, Smit JJ, van Tellingen O, et al. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell*. 1994;77:491-502.
6. Kyle AH, Huxham LA, Yeoman DM, et al. Limited tissue penetration of taxanes: a mechanism for resistance in solid tumors. *Clin Cancer Res*. 2007;13:2804-2810.
7. Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer*. 2006;6:583-592.
8. Matsumoto S, Batra S, Saito K, et al. Antiangiogenic agent sunitinib transiently increases tumor oxygenation and suppresses cycling hypoxia. *Cancer Res*. 2011;71:6350-6359.
9. Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med*. 2002;53:615-627.
10. Quintieri L, Fantin M, Vizler C. Identification of molecular determinants of tumor sensitivity and resistance to anticancer drugs. *Adv Exp Med Biol*. 2007;593:95-104.
11. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5:275-284.
12. Clarke AR, Purdie CA, Harrison DJ, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*. 1993;362:849-852.
13. Fisher TC, Milner AE, Gregory CD, et al. bcl-2 modulation of apoptosis induced by anticancer drugs: resistance to thymidylate stress is independent of classical resistance pathways. *Cancer Res*. 1993;53:3321-3326.
14. Miyashita T, Reed JC. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res*. 1992;52:5407-5411.
15. Fan S, el-Deiry WS, Bae I, et al. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res*. 1994;54:5824-5830.
16. Reed JC. Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. *Semin Hematol*. 1997;34:9-19.
17. Strasser A, Huang DC, Vaux DL. The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumourigenesis and resistance to chemotherapy. *Biochim Biophys Acta*. 1997;1333:F151-F178.
18. Schmitt CA, Lowe SW. Apoptosis and therapy. *J Pathol*. 1999;187:127-137.
19. Inoue S, Salah-Eldin AE, Omoteyama K. Apoptosis and anticancer drug resistance. *Hum Cell*. 2001;14:211-221.
20. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell*. 2002;108:153-164.
21. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*. 2006;25:4798-4811.
22. Lowe SW, Schmitt EM, Smith SW, et al. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*. 1993;362:847-849.
23. Lowe SW, Ruley HE, Jacks T, et al. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*. 1993;74:957-967.
24. Boise LH, Gonzalez-Garcia M, Postema CE, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 1993;74:597-608.
25. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*. 1993;81:151-157.
26. Morin PJ. Drug resistance and the microenvironment: nature and nurture. *Drug Resist Updat*. 2003;6:169-172.
27. Fojo T. Multiple paths to a drug resistance phenotype: mutations, translocations, deletions and amplification of coding genes or promoter regions, epigenetic changes and microRNAs. *Drug Resist Updat*. 2007;10:59-67.
28. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976;194:23-28.
29. Greaves M, Maley CC. Clonal evolution in cancer. *Nature*. 2012;481:306-313.
30. Goldie JH, Coldman AJ. A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. *Cancer Treat Rep*. 1979;63:1727-1733.
31. Goldie JH, Coldman AJ. Genetic instability in the development of drug resistance. *Semin Oncol*. 1985;12:2222-2230.
32. Woodhouse JR, Ferry DR. The genetic basis of resistance to cancer chemotherapy. *Ann Med*. 1995;27:157-167.
33. Jaffrezou JP, Chen G, Duran GE, et al. Mutation rates and mechanisms of resistance to etoposide determined from fluctuation analysis. *J Natl Cancer Inst*. 1994;86:1152-1158.
34. Chen G, Jaffrezou JP, Fleming WH, et al. Prevalence of multidrug resistance related to activation of the mdr1 gene in human sarcoma mutants derived by single-step doxorubicin selection. *Cancer Res*. 1994;54:4980-4987.
35. Beketic-Oreskovic L, Duran GE, Chen G, et al. Decreased mutation rate for cellular resistance to doxorubicin and suppression of mdr1 gene activation by the cyclosporin PSC 833. *J Natl Cancer Inst*. 1995;87:1593-1602.
36. Dumontet C, Duran GE, Steger KA, et al. Resistance mechanisms in human sarcoma mutants derived by single-step exposure to paclitaxel (Taxol). *Cancer Res*. 1996;56:1091-1097.
37. Matsumoto Y, Takano H, Fojo T. Cellular adaptation to drug exposure: evolution of the drug-resistant phenotype. *Cancer Res*. 1997;57:5086-5092.
38. Chen KG, Wang YC, Schaner ME, et al. Genetic and epigenetic modeling of the origins of multidrug-resistant cells in a human sarcoma cell line. *Cancer Res*. 2005;65:9388-9397.
39. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366:883-892.
40. Lee AJ, Swanton C. Tumour heterogeneity and drug resistance: personalising cancer medicine through functional genomics. *Biochem Pharmacol*. 2012;83:1013-1020.
41. Swanton C. Intratumor heterogeneity: evolution through space and time. *Cancer Res*. 2012;72:4875-4882.
42. Alt FW, Kellems RE, Bertino JR, et al. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem*. 1978;253:1357-1370.
43. Wang YC, Juric D, Francisco B, et al. Regional activation of chromosomal arm 7q with and without gene amplification in taxane-selected human ovarian cancer cell lines. *Genes Chromosomes Cancer*. 2006;45:365-374.
44. Matei D, Fang F, Shen C, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res*. 2012;72:2197-2205.
45. Hill BT, Deuchars K, Hosking LK, et al. Overexpression of P-glycoprotein in mammalian tumor cell lines after fractionated X irradiation in vitro. *J Natl Cancer Inst*. 1990;82:607-612.
46. Brugger D, Brischwein K, Liu C, et al. Induction of drug resistance and protein kinase C genes in A2780 ovarian cancer cells after incubation with antineoplastic agents at sublethal concentrations. *Anticancer Res*. 2002;22:4229-4232.
47. Abolhoda A, Wilson AE, Ross H, et al. Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clin Cancer Res*. 1999;5:3352-3356.
48. Wilting RH, Dannenberg JH. Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance. *Drug Resist Updat*. 2012;15:21-38.
49. Balch C, Nephew KP. Epigenetic targeting therapies to overcome chemotherapy resistance. *Adv Exp Med Biol*. 2013;754:285-311.
50. Zeller C, Dai W, Steele NL, et al. Candidate DNA methylation drivers of acquired cisplatin resistance in ovarian cancer identified by methylome and expression profiling. *Oncogene*. 2012;31:4567-4576.
51. Shen DW, Pouliot LM, Hall MD, et al. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. *Pharmacol Rev*. 2012;64:706-721.
52. Bhatla T, Wang J, Morrison DJ, et al. Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. *Blood*. 2012;119:5201-5210.

53. Chen KG, Sikic BI. Molecular pathways: regulation and therapeutic implications of multidrug resistance. *Clin Cancer Res*. 2012;18:1863-1869.
54. Rosenzweig SA. Acquired resistance to drugs targeting receptor tyrosine kinases. *Biochem Pharmacol*. 2012;83:1041-1048.
55. Sharma SV, Lee DY, Li B, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell*. 2010;141:69-80.
56. Mongroo PS, Rustgi AK. The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol Ther*. 2010;10:219-222.
57. Basile KJ, Aplin AE. Resistance to chemotherapy: short-term drug tolerance and stem cell-like subpopulations. *Adv Pharmacol*. 2012;65:315-334.
58. Ricci F, Bernasconi S, Perego P, et al. Ovarian carcinoma tumor-initiating cells have a mesenchymal phenotype. *Cell Cycle*. 2012;11:1966-1976.
59. Smalley M, Piggott L, Clarkson R. Breast cancer stem cells: Obstacles to therapy. *Cancer Lett*. 2012 Apr 30 [Epub ahead of print].
60. Maugeri-Sacca M, Vigneri P, De Maria R. Cancer stem cells and chemosensitivity. *Clin Cancer Res*. 2011;17:4942-4947.
61. Sebens S, Schafer H. The tumor stroma as mediator of drug resistance—a potential target to improve cancer therapy? *Curr Pharm Biotechnol*. 2012;13:2259-2272.
62. Sun Y, Nelson PS. Molecular pathways: involving microenvironment damage responses in cancer therapy resistance. *Clin Cancer Res*. 2012;18:4019-4025.
63. Westhoff MA, Fulda S. Adhesion-mediated apoptosis resistance in cancer. *Drug Resist Updat*. 2009;12:127-136.
64. Flach EH, Rebecca VW, Herlyn M, et al. Fibroblasts contribute to melanoma tumor growth and drug resistance. *Mol Pharm*. 2011;8:2039-2049.
65. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. *J Bioenerg Biomembr*. 2001;33:453-458.
66. Huang Y. Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis Rev*. 2007;26:183-201.
67. Glavinas H, Krajcsi P, Cserepes J, et al. The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr Drug Deliv*. 2004;1:27-42.
68. Kruh GD, Zeng H, Rea PA, et al. MRP subfamily transporters and resistance to anticancer agents. *J Bioenerg Biomembr*. 2001;33:493-501.
69. Riordan JR, Ling V. Genetic and biochemical characterization of multidrug resistance. *Pharmacol Ther*. 1985;28:51-75.
70. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, et al. P-glycoprotein: from genomics to mechanism. *Oncogene*. 2003;22:7468-7485.
71. Safa AR, Glover CJ, Meyers MB, et al. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *J Biol Chem*. 1986;261:6137-6140.
72. Ueda K, Cardarelli C, Gottesman MM, et al. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci U S A*. 1987;84:3004-3008.
73. Thiebaut F, Tsuruo T, Hamada H, et al. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A*. 1987;84:7735-7738.
74. Shukla S, Chen ZS, Ambudkar SV. Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance. *Drug Resist Updat*. 2012;15:70-80.
75. Dalton WS, Grogan TM, Meltzer PS, et al. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol*. 1989;7:415-424.
76. Campos L, Guyotat D, Archimbaud E, et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. *Blood*. 1992;79:473-476.
77. Miller TP, Grogan TM, Dalton WS, et al. P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. *J Clin Oncol*. 1991;9:17-24.
78. Marie JP, Zittoun R, Sikic BI. Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity. *Blood*. 1991;78:586-592.
79. Bradshaw DM, Arceci RJ. Clinical relevance of transmembrane drug efflux as a mechanism of multidrug resistance. *J Clin Oncol*. 1998;16:3674-3690.
80. Mahadevan D, List AF. Targeting the multidrug resistance-1 transporter in AML: molecular regulation and therapeutic strategies. *Blood*. 2004;104:1940-1951.
81. Clarke R, Leonessa F, Trock B. Multidrug resistance/P-glycoprotein and breast cancer: review and meta-analysis. *Semin Oncol*. 2005;32:S9-S15.
82. Sikic BI. Modulation of multidrug resistance: at the threshold. *J Clin Oncol*. 1993;11:1629-1635.
83. Fisher GA, Sikic BI. Clinical studies with modulators of multidrug resistance. *Hematol Oncol Clin North Am*. 1995;9:363-382.
84. Sikic BI. Pharmacologic approaches to reversing multidrug resistance. *Semin Hematol*. 1997;34:40-47.
85. Lum BL, Kaubisch S, Yahanda AM, et al. Alteration of etoposide pharmacokinetics and pharmacodynamics by cyclosporine in a phase I trial to modulate multidrug resistance. *J Clin Oncol*. 1992;10:1635-1642.
86. Lum BL, Kaubisch S, Fisher GA, et al. Effect of high-dose cyclosporine on etoposide pharmacodynamics in a trial to reverse P-glycoprotein (MDR1 gene) mediated drug resistance. *Cancer Chemother Pharmacol*. 2000;45:305-311.
87. Advani R, Fisher GA, Lum BL, et al. A phase I trial of doxorubicin, paclitaxel, and valspodar (PSC 833), a modulator of multidrug resistance. *Clin Cancer Res*. 2001;7:1221-1229.
88. List AF, Kopecky KJ, Willman CL, et al. Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology. Group study. *Blood*. 2001;98:3212-3220.
89. Dantzig AH, Law KL, Cao J, et al. Reversal of multidrug resistance by the P-glycoprotein modulator, LY335979, from the bench to the clinic. *Curr Med Chem*. 2001;8:39-50.
90. Loe DW, Deeley RG, Cole SP. Biology of the multidrug resistance-associated protein, MRP. *Eur J Cancer*. 1996;32A:945-957.
91. Muller M, Meijer C, Zaman GJ, et al. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci U S A*. 1994;91:13033-13037.
92. Jedlitschky G, Leier I, Buchholz U, et al. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochem J. 327 part*. 1997;1:305-310.
93. Kool M, van der Linden M, de Haas M, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci U S A*. 1999;96:6914-6919.
94. Loe DW, Deeley RG, Cole SP. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. *Cancer Res*. 1998;58:5130-5136.
95. Keppler D, Leier I, Jedlitschky G. Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol Chem*. 1997;378:787-791.
96. Leier I, Jedlitschky G, Buchholz U, et al. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem*. 1994;269:27807-27810.
97. Morrow CS, Smitherman PK, Diah SK, et al. Coordinated action of glutathione S-transferases (GSTs) and multidrug resistance protein 1 (MRP1) in antineoplastic drug detoxification. Mechanism of GST A1-1- and MRP1-associated resistance to chlorambucil in MCF7 breast carcinoma cells. *J Biol Chem*. 1998;273:20114-20120.
98. Fukuda Y, Schuetz JD. ABC transporters and their role in nucleoside and nucleotide drug resistance. *Biochem Pharmacol*. 2012;83:1073-1083.
99. Ross DD, Yang W, Abruzzo LV, et al. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. *J Natl Cancer Inst*. 1999;91:429-433.
100. Bates SE, Robey R, Miyake K, et al. The role of half-transporters in multidrug resistance. *J Bioenerg Biomembr*. 2001;33:503-511.
101. Benderra Z, Faussat AM, Sayada L, et al. Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. *Clin Cancer Res*. 2004;10:7896-7902.
102. Benderra Z, Faussat AM, Sayada L, et al. MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. *Clin Cancer Res*. 2005;11:7764-7772.
103. Plasschaert SL, Van Der Kolk DM, De Bont ES, et al. Breast cancer resistance protein (BCRP) in acute leukemia. *Leuk Lymphoma*. 2004;45:649-654.
104. Raaijmakers MH, de Grouw EP, Heuver LH, et al. Breast cancer resistance protein in drug resistance of primitive CD34+38- cells in acute myeloid leukemia. *Clin Cancer Res*. 2005;11:2436-2444.

105. Sikic BI. Multidrug resistance and stem cells in acute myeloid leukemia. *Clin Cancer Res*. 2006;12:3231-3232.
106. van der Holt B, Van den Heuvel-Eibrink MM, Van Schaik RH, et al. ABCB1 gene polymorphisms are not associated with treatment outcome in elderly acute myeloid leukemia patients. *Clin Pharmacol Ther*. 2006;80:427-439.
107. Safaei R, Howell SB. Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs. *Crit Rev Oncol Hematol*. 2005;53:13-23.
108. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov*. 2005;4:307-320.
109. Moscow JA. Methotrexate transport and resistance. *Leuk Lymphoma*. 1998;30:215-224.
110. Perez RP. Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer*. 1998;34:1535-1542.
111. Haber DA, Schimke RT. Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. *Cell*. 1981;26:355-362.
112. Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol*. 1999;17:1061-1070.
113. Orr GA, Verdier-Pinard P, McDaid H, et al. Mechanisms of taxol resistance related to microtubules. *Oncogene*. 2003;22:7280-7295.
114. Séve P, Mackey J, Isaac S, et al. Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. *Mol Cancer Ther*. 2005;4:2001-2007.
115. Yusuf RZ, Duan Z, Lamendola DE, et al. Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. *Curr Cancer Drug Targets*. 2003;3:1-19.
116. Sale S, Sung R, Shen P, et al. Conservation of the class I beta tubulin gene in human populations and lack of mutations in lung cancers and paclitaxel resistant ovarian cancers. *Mol Cancer Ther*. 2002;1:215-225.
117. Mesquita B, Veiga I, Pereira D, et al. No significant role for beta tubulin mutations and mismatch repair defects in ovarian cancer resistance to paclitaxel/cisplatin. *BMC Cancer*. 2005;5:101-106.
118. Rouzier R, Rajan R, Wagner P, et al. Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer. *Proc Natl Acad Sci U S A*. 2005;102:8315-8320.
119. Wagner P, Wang B, Clark E, et al. Microtubule Associated Protein (MAP)-Tau: a novel mediator of paclitaxel sensitivity in vitro and in vivo. *Cell Cycle*. 2005;4:1149-1152.
120. Sudo T, Nitta M, Saya H, et al. Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Res*. 2004;64:2502-2508.
121. Sugimura M, Sagae S, Ishioka S, et al. Mechanisms of paclitaxel-induced apoptosis in an ovarian cancer cell line and its paclitaxel-resistant clone. *Oncology*. 2004;66:53-61.
122. Goodin S, Kane MP, Rubin EH. Epothilones: mechanism of action and biologic activity. *J Clin Oncol*. 2004;22:2015-2025.
123. Lee JJ, Swain SM. Development of novel chemotherapeutic agents to evade the mechanisms of multidrug resistance (MDR). *Semin Oncol*. 2005;32:S22-S26.
124. Andoh T, Ishii K, Suzuki Y, et al. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc Natl Acad Sci U S A*. 1987;84:5565-5569.
125. Deffie AM, Batra JK, Goldenberg GJ. Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines. *Cancer Res*. 1989;49:58-62.
126. Tanizawa A, Pommier Y. Topoisomerase I alteration in a camptothecin-resistant cell line derived from Chinese hamster DC3F cells in culture. *Cancer Res*. 1992;52:1848-1854.
127. Beck WT, Morgan SE, Mo YY, et al. Tumor cell resistance to DNA topoisomerase II inhibitors: new developments. *Drug Resist Updat*. 1999;2:382-389.
128. Xu Y, Villalona-Calero MA. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol*. 2002;13:1841-1851.
129. Rubin EH, Li TK, Duann P, et al. Cellular resistance to topoisomerase poisons. *Cancer Treat Res*. 1996;87:243-260.
130. Lackner MR, Wilson TR, Settleman J. Mechanisms of acquired resistance to targeted cancer therapies. *Future Oncol*. 2012;8:999-1014.
131. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance and the road to a cure of chronic myeloid leukemia. *Blood*. 2007;110:2242-2249.
132. Diamond JM, Melo JV. Mechanisms of resistance to BCR-ABL kinase inhibitors. *Leuk Lymphoma*. 2011;52(Suppl 1):12-22.
133. Kickhoefer VA, Rajavel KS, Scheffer GL, et al. Vaults are up-regulated in multidrug-resistant cancer cell lines. *J Biol Chem*. 1998;273:8971-8974.
134. List AF, Spier CS, Grogan TM, et al. Overexpression of the major vault transporter protein lung-resistance protein predicts treatment outcome in acute myeloid leukemia. *Blood*. 1996;87:2464-2469.
135. Steuart CD, Burke PJ. Cytidine deaminase and the development of resistance to arabinosyl cytosine. *Nat New Biol*. 1971;233:109-110.
136. Milano G, McLeod HL. Can dihydropyrimidine dehydrogenase impact 5-fluorouracil-based treatment? *Eur J Cancer*. 2000;36:37-42.
137. Sikic BI. Biochemical and cellular determinants of bleomycin cytotoxicity. *Cancer Surv*. 1986;5:81-91.
138. Black SM, Wolf CR. The role of glutathione-dependent enzymes in drug resistance. *Pharmacol Ther*. 1991;51:139-154.
139. Buller AL, Clapper ML, Tew KD. Glutathione S-transferases in nitrogen mustard-resistant and -sensitive cell lines. *Mol Pharmacol*. 1987;31:575-578.
140. Robson CN, Lewis AD, Wolf CR, et al. Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione S-transferase activity. *Cancer Res*. 1987;47:6022-6027.
141. Kramer RA, Zakher J, Kim G. Role of the glutathione redox cycle in acquired and de novo multidrug resistance. *Science*. 1988;241:694-697.
142. Lewis AD, Hickson ID, Robson CN, et al. Amplification and increased expression of alpha class glutathione S-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc Natl Acad Sci U S A*. 1988;85:8511-8515.
143. Moscow JA, Townsend AJ, Cowan KH. Elevation of pi class glutathione S-transferase activity in human breast cancer cells by transfection of the GST pi gene and its effect on sensitivity to toxins. *Mol Pharmacol*. 1989;36:22-28.
144. Sinha BK, Mimnaugh EG, Rajagopalan S, et al. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. *Cancer Res*. 1989;49:3844-3848.
145. Morrow CS, Cowan KH. Glutathione S-transferases and drug resistance. *Cancer Cell*. 1990;2:15-22.
146. Dirven HA, van Ommen B, van Bladeren PJ. Involvement of human glutathione S-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. *Cancer Res*. 1994;54:6215-6220.
147. Tew KD. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res*. 1994;54:4313-4320.
148. Dirven HA, Dictus EL, Broeders NL, et al. The role of human glutathione S-transferase isoenzymes in the formation of glutathione conjugates of the alkylating cytostatic drug thiotepa. *Cancer Res*. 1995;55:1701-1706.
149. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer*. 2004;4:296-307.
150. Zhang J, Tian Q, Chan SY, et al. Insights into oxazaphosphorine resistance and possible approaches to its circumvention. *Drug Resist Updat*. 2005;8:271-297.
151. Kaina B, Christmann M. DNA repair in resistance to alkylating anticancer drugs. *Int J Clin Pharmacol Ther*. 2002;40:354-367.
152. Zamble DB, Lippard SJ. Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem Sci*. 1995;20:435-439.
153. Ceppi P, Volante M, Novello S, et al. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. *Ann Oncol*. 2006;17:1818-1825.
154. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*. 2006;355:983-991.
155. Zheng Z, Chen T, Li X, et al. DNA synthesis and repair genes RRM1 and ERCC1 in lung cancer. *N Engl J Med*. 2007;356:800-808.
156. Gazdar AF. DNA repair and survival in lung cancer—the two faces of Janus. *N Engl J Med*. 2007;356:771-773.
157. Spears CP. Clinical resistance to antimetabolites. *Hematol Oncol Clin North Am*. 1995;9:397-413.

158. Houghton JA, Maroda Jr SJ, Phillips JO, et al. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. *Cancer Res*. 1981;41:144-149.
159. Smolewski P, Robak T. Inhibitors of apoptosis proteins (IAPs) as potential molecular targets for therapy of hematological malignancies. *Curr Mol Med*. 2011;11:633-649.
160. Jendrossek V. The intrinsic apoptosis pathways as a target in anticancer therapy. *Curr Pharm Biotechnol*. 2012;13:1426-1438.
161. Gianni L, Zambetti M, Clark K, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. *J Clin Oncol*. 2005;23:7265-7277.
162. Hess KR, Anderson K, Symmans WF, et al. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. *J Clin Oncol*. 2006;24:4236-4244.

Daniel Morgensztern, Patricia M. LoRusso, Scott A. Boerner,
Roy S. Herbst, and Joseph Paul Eder

48 Phase I Trials Today

Introduction

All commercially available anticancer agents must have undergone Phase I investigation as part of their clinical development. As novel anticancer drugs evolved from primarily cytotoxic agents to targeted therapies, clinical investigators have developed novel Phase I trial designs and endpoints. It is estimated that approximately 500 anticancer agents will present to the clinical arena within the next decade. It is a well-known fact that one of the most important components of conducting Phase I trials is eligible patient availability. As the number of commercially available agents for several tumor types has increased, as well as the number of patients treated off-protocol in community settings, the availability of patient resources has become a challenge.

As a result, it is important to conduct efficient and effective trials by maximizing data acquisition while minimizing patient numbers. Previously, standard Phase I trials used large patient numbers and cohorts. Now, it is the norm to utilize well-thought-out trials minimizing patient numbers and cohorts by using alternative designs and carefully selecting the starting dose. Once thought of only as an alternative to hospice with no significant benefit, treatment on a Phase I trial is now viewed as an additional therapeutic option. The overall clinical benefit of Phase I trials is approximately 45%, with highly variable response rates, depending on the type of agent and the Phase I trial under investigation.[1] Ethically, the intent of all clinical studies, for both the patient and physician alike, is therapeutic.[2-4] A better understanding of the compound(s) under investigation and the various types of Phase I clinical trials available will assist the investigator in determining at what point and for which patient specific Phase I clinical trials should be considered.

Types of Phase I Clinical Trials

Phase I clinical trials are the first stage of drug testing in human subjects. These studies play a vital role in the development of novel therapeutics. Phase I studies are typically designed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of a novel agent. Novel cancer therapeutics are usually offered to patients with advanced cancer who have had other types of therapies and who have few, if any, remaining treatment options. In addition, because of several tumor types with limited current treatment options that could have a favorable impact on patient survival, it is considered ethical to treat patients with metastatic disease in Phase I trials by utilizing novel agents in combination with standard therapies; this is especially true if the standard therapies demonstrated therapeutic success in previous clinical investigations.

Although the primary stated objective of a typical Phase I study is to determine the optimal dose of a novel therapeutic for use in subsequent studies, several different types of Phase I clinical trials exist to meet specific needs for clinical early drug development.

Single Ascending Dose (SAD)

Single ascending dose (SAD) studies are those in which groups, or cohorts, of up to six patients are given a small dose of the drug and observed for a specific period of time. The initial dose is commonly based on one tenth of the murine equivalent lethal dose in 10% of animals (MELD10) or the human equivalent dose of the no observed adverse effect level (NOAEL, the highest nontoxic dose) achieved in the most sensitive preclinical species multiplied by a safety factor (default is greater than 10-fold). If the cohort does not exhibit any adverse side effects, a new group of patients is then given a higher dose. This continues until intolerable side effects are observed. Once such side effects occur in a patient, the protocol typically explores whether this is sporadic or reproducible, as described later in detail. The highest dose administered to a patient on a Phase I trial is referred to as the *maximum administered dose*. The dose that is as high as possible but still tolerable for patients is said to be the *maximum tolerated dose* (MTD).

Often, SAD clinical trials can be categorized as being first-in-human, first-in-class, or a combination of the two. As the name implies, first-in-human clinical trials are those that are conducted for the first time in a human patient. In order to be tested in humans, a drug typically has to first show promise of activity in the laboratory and in animals. Normally, a small group of patients (approximately 20) will be selected for inclusion into a first-in-human Phase I study. First-in-human studies for noncancer indications are almost always done in a single ascending dose manner. The objective of the first-in-human Phase I trial is to find a suitable safe dose (the MTD) for use in later studies that will more thoroughly examine efficacy. Once the MTD has been determined in a Phase I SAD study, later phase studies can be designed and multiple ascending dose studies can be performed.

First-in-class studies examine novel drugs that are uniquely manufactured or based on a new target or indication. Such therapeutics are typically innovative and novel, and no other pharmaceutical products are currently approved for the same therapeutic indication; hence, they have no pharmaceutical substitute.

Multiple Ascending Dose (MAD)

Multiple ascending dose (MAD) studies are conducted to better understand the pharmacokinetics and pharmacodynamics of a drug and determine the dose that is tolerable for repeated administration in therapeutic intent trials (Phase II). In these studies, a group of patients receives a low dose of the drug and the dose is subsequently escalated to a predetermined level. A single schedule that is judged optimal from preclinical studies or multiple schedules may be tested. Specimens (of blood and/or other fluids) are collected at various time points and analyzed to understand how the drug is processed within the body. In cancer, where MAD studies are commonplace, subjects receive repeated doses at a predetermined schedule, and new cohorts of patients receive progressively higher doses on the same schedule (see later discussion).

Method and Model (MeMo)

MeMo trials are studies that are done in anticipation of a Phase I clinical trial. Typically done for "targeted" agents, these trials help in the development of a pharmacodynamic endpoint. They may help identify either a direct tissue or a surrogate tissue marker. This assists in determining if the marker can be measured within the tissue and also helps to refine the assay needed for pharmacodynamic measurement.

Phase I Trials Using Radiolabeled Tracer Doses

The use of radiolabeled experimental agents has become an increasingly important factor in drug development. In preclinical studies, radiolabeled compounds are frequently used in the laboratory to understand the distribution, metabolic fate, and localization of experimental drugs both in vitro and in vivo. Clinical studies performed as part of Phase I trials, or in support of them, may also involve the administration of small doses of radiolabeled compounds, called *tracers*, to healthy human volunteers or to patients in order to better understand the mechanisms of drug action.

Radiolabeled tracers are synthesized by replacing one or more atoms of an experimental drug agent with a radioisotope. Radioisotopes must have a suitable half-life in order to allow for imaging or detection in biological samples. Examples of commonly used isotopes for detection in tissue or blood samples include carbon (^{14}C), hydrogen (^{3}H), sulfur (^{35}S), and iodine (^{125}I). Isotopes that are commonly used in imaging, specifically in positron emission tomography (PET) scanning, include fluorine (^{18}F), carbon (^{11}C), and oxygen (^{15}O).

Radiolabeled compounds have allowed researchers to study many aspects of a drug's behavior in vivo.[5] Evaluation of the mass balance of a drug can be performed to better understand how much of an applied dose is recovered with respect to time. The metabolism of the drug can be extensively studied to determine if any metabolites might represent a potential toxicological hazard to the patient. Advances in clinical imaging have had great impact on drug discovery and development in recent years.[6] Clinical imaging studies using labeled drug have the potential to facilitate early clinical pharmacokinetic/pharmacodynamic assessments, including target interaction and modulation.[7] This is particularly useful in patients where there are no direct measures of pharmacokinetics/pharmacodynamics throughout the tissues of the body and at the target.

Studies using a method called *microdosing* offer the prospect of taking a drug directly into human studies by administering extremely low doses of radiolabeled agent. Microdosing studies may also be referred to as Phase 0 studies. Using only tiny amounts of radiolabeled drug, researchers employ microdosing to establish the likely pharmacological dose and thereby determine the first dose for a subsequent Phase I study. However, microdosing is not without controversy among researchers in drug development.[5] Concern has been raised that microdosing may not accurately predict the behavior of clinical doses. It has also been suggested that nonlinearities may be induced when binding, metabolizing, or eliminating systems become saturated, thus resulting in differences between low and high doses.

Drug/Food Metabolic Interaction Studies

The U.S. Food and Drug Administration (FDA) has recommended that the metabolism of an investigational new drug be defined during drug development and that interactions with other drugs be explored as part of an adequate assessment of its safety and effectiveness.[8] Medicines are often used concomitantly with other drugs, and some degree of drug-drug interaction often occurs with concomitant use. Concomitant medications can abruptly alter metabolic routes of absorption and elimination. Although only a small proportion of drug interactions are clinically significant, they sometimes cause serious adverse reactions.

Therefore, early on in the drug development process, appropriate efforts should be made to predict the nature and degree of potential interactions so that patients will not be adversely affected. The important cytochrome P450 (CYP450) family of enzymes is found in the liver and plays a large role in metabolizing drugs. Many metabolic routes of elimination, including most of those occurring via the CYPP450 family of enzymes, can be inhibited, activated, or induced by concomitant drug treatment. The FDA has recommended that detailed studies be performed with the major CYP450 enzymes (CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4). Typically, preclinical testing is performed to investigate the effects of an agent on metabolic factors, such as CYP450, and of inhibition or induction potential. If in vitro experiments reveal the potential for drug-drug interaction, in vivo experiments usually will follow. Therefore, Phase I clinical trials often include testing for the ability of an experimental agent to affect CYP450 and a determination of whether the agent causes a change in concentration of other drugs as a result. With the combination of in vitro studies and in vivo studies in support of Phase I clinical trials, the potential for drug-drug interactions can be studied early in the development process, with further study of observed interactions assessed later in the process, if necessary.

Organ Dysfunction Studies

The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations (accumulation), which are affected by its absorption, distribution, metabolism, and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver, or by excretion, usually by the kidneys and liver.

Although clinical trials for drug approval are often conducted in patients with normal hepatic and renal function, patients in clinical practice, especially those with cancer, may have compromised organ function because of underlying disease.[8] It has been recommended that organ dysfunction studies be designed in the form of a formal dose-escalation Phase I study, with a complete pharmacokinetic and toxicity profile as endpoints.[8,9] The primary goal of the Phase I study in an organ-impaired population should be to determine if the pharmacokinetics are altered to such an extent that the dosage requires adjustment, based on degree of organ dysfunction, from the dose established in the unimpaired population.

Because of the uniqueness of eligible patients, these studies are typically conducted as multisite studies so that they can be completed in a timely and efficient fashion. In 1999, the National Cancer Institute developed an Organ Dysfunction Working Group (ODWG), comprising approximately 12 to 15 Phase I sites. The ODWG has successfully completed evaluation of oxaliplatin, imatinib, and bortezomib in the renally and hepatically impaired populations.[10-15] In addition, several additional agents are currently undergoing evaluation.

Thorough QT Phase I Studies

Adverse effects on cardiac health have become one of the most common causes of product withdrawal from the market. As a result, regulatory authorities around the world have recently placed greater emphasis on cardiac safety. The FDA's regulatory guidance recommends a thorough QT Phase I study to be conducted irrespective of preclinical cardiac findings.[16] When a thorough QT study is not feasible for other reasons, which may be the case in certain therapeutic areas such as oncology, alternative approaches are recommended, such as expanding the number and timings of electrocardiographic (ECG) recordings in other clinical studies in patients.

Dose-Scheduling Studies

Inefficient dose scheduling can lead to treatment failure and the inadequate development of a potentially promising therapeutic. Unlike the typical Phase I clinical trial designed to determine the MTD, an investigator may be interested in determining how often (i.e., how many administrations of a schedule) an agent could be safely administered to determine the long-term toxicity due to cumulative effects. Dose-scheduling studies are designed to determine the optimal administration schedule for an investigational agent.

Dose-scheduling studies can be combined with dose-finding studies or can be completely separate studies. Pharmacokinetic and safety data obtained during a Phase I

dose-finding study may suggest it is feasible to increase the dose and/or reduce the frequency of administration of an agent, therefore indicating that a dose-scheduling study is warranted.

Combination Studies

The typical Phase I dose-finding study is designed to determine the maximally tolerated dose of a single, novel agent. However, an increasing number of patients, particularly in oncology, are being treated with drug combinations. The goal of a two-agent dose-finding trial is to find the maximally tolerated dose of a dose combination (or combinations). Combination studies can be performed to determine the optimal dose and schedule of experimental drugs combined with standard chemotherapies and also of novel drugs combined together.

At the time that a combination study is designed, the monotherapy MTDs of the individual agents under investigation are usually known. As a result, a minimal number of dose levels are typically needed to achieve the recommended dose of both drugs in combination. In the past, combination studies were routinely performed as single-arm trials. However, recent novel designs, which include targeted and standard therapies, have been designed with several arms with differing standard therapies in combination with the novel agent under investigation.[17] Such a design has been shown to expedite the identification of the combination MTD.

A common design for dose-finding studies with multiple agents is to investigate a single dose, or a small number of doses, of one agent and multiple doses of the second agent. If the study is combining a novel agent with a standard chemotherapy, the dose of the novel agent is usually varied while the standard chemotherapy is held to a single or a few doses.

Phase I Cancer Clinical Trial Designs

Many Phase I clinical trial design methods have been proposed, and there is currently no consensus among the scientific, medical, and statistical communities on how best to perform these studies in humans.

Traditional Design

The most commonly used design, often referred to as the *traditional* or *3+3 design*, begins by assigning three patients in a cohort at a designated dose level, often one-tenth the lethal dose (LD_{10}) in mice or the NOAEL in the most sensitive animal species, scaled to humans.[18] Doses to be assigned are predefined by the investigators, based on preclinical data and clinical experience with similar agents, if it exists. One method of assigning successive dose levels uses a set of "increasing decreasing" Fibonacci dose-level increments, usually 100%, 67%, 50%, 40%, and 33% for each dose level thereafter.[19-21] These increments are added to each dose to get the next dose level. For example, the second dose level is 100% more than the first, the third dose level is 67% more than the second, the fourth dose level is 50% more than the third, and so on.

The decision whether to escalate to the next higher dose, expand a cohort, or de-escalate to a lower dose is made based on the toxicity information received from each three-patient cohort (Figure 48-1). If none of the three patients experiences a dose-limiting toxicity (DLT), the study proceeds to another cohort of three patients at a higher dose level (escalation phase). DLTs are predefined and often include unacceptable drug-related toxicities as defined in grading scales commonly used in oncology, such as the National Cancer Institute's Common Terminology

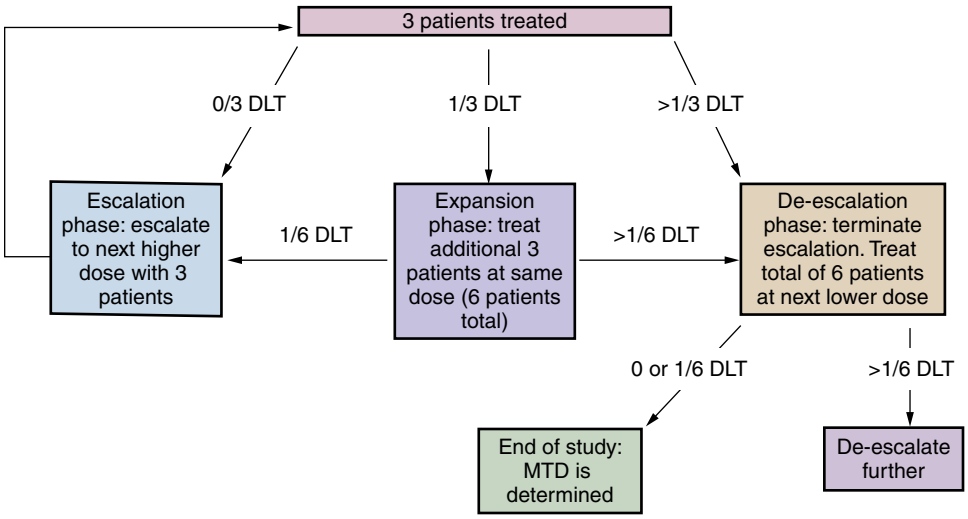


Figure 48-1 The traditional "3 + 3" Phase I clinical trial design. The initial 3-patient cohort begins at a predefined dose. If no DLTs are observed, escalation to the next higher dose will occur. If a single DLT is observed, expansion of the cohort to a total of six patients occurs. If more than 1 DLT is observed, de-escalation to the next lowest dose will occur for a total of six patients treated at that dose. Termination of the study will occur if more than one DLT is observed at the starting dose. The MTD is defined as the highest dose level for which no more than one patient out of six experiences a DLT.

Criteria for Adverse Events (CTCAE). If 1 of the 3 patients treated on a dose-level cohort experiences a DLT, up to an additional 3 patients (for a total of 6) are treated at that dose level (expansion phase). If none of the additional patients experiences a DLT, the dose will escalate. If at least 2 patients experience a DLT, the MTD is said to have been exceeded and the maximum administered dose (MAD) has been defined. An additional 3 patients will be tested at the next lowest dose level if there were only 3 patients previously treated at that level (de-escalation phase). In this particular case, the MTD is therefore defined as the highest dose level for which no more than 1 patient in 6 experiences a DLT. Table 48-1 shows the different dose escalation/deescalation decisions associated with toxicity outcomes at a given dose for an example of the 3+3 design.

The rolling six design (RSD) has been used by the Children's Oncology Group as an alternative design to the 3+3 design.[22,23] Because ethical considerations of informed consent almost always preclude a first-in-human trial in a pediatric population, the RSD is used to reduce trial interruptions and delays where there is prior human experience. The RSD begins like the 3+3 design and enrolls a cohort of 3 subjects on a dose. A fourth subject can be enrolled if at least one of the first 3 patients has not fully completed the initial first-dose evaluation for toxicity and no more than 1 of the previous 3 subjects has experienced a DLT. A fifth (sixth) patient can be enrolled if at least 1 of the first 4 (5) subjects has not completed the initial observation period and no more than 1 DLT has been observed. If not more than 1 fully evaluable patient at a specific dose cohort experiences a DLT, the study enrolls a new cohort of patients on the next highest dose. If at any time, 2 of 2 to 6 patients receiving the same dose experience a DLT, the study enrolls a new cohort of subjects on the next lowest dose. If 6 patients were already entered at the next lowest dose, that next lowest dose is selected as the MTD. The RSD allows for temporal overlap of the two cohorts of 3 subjects used in the 3+3 design. Hence, the probability of trial suspension to further accrual is lower in the RSD design as compared to the 3+3 design.[22]

Table 48-1 Dose Escalation/De-escalation Decisions Associated with Toxicity Outcomes at a Given Dose for a Popular Version of the 3 + 3 Design

No. of Patients with Dose-Limiting Toxicity	Decision
0/3	Escalate one level
1/3	Treat 3 more at same level
1/3 + 0/3*	Escalate one level
1/3 + 1/3*	Stop and choose previous dose as the MTD
1/3 + (2/3 or 3/3)*	Stop and choose previous dose as the MTD
2/3 or 3/3	Stop and choose previous dose as the MTD

*Note that those rows with number of toxicities equal to 1/3 + t/3 (for t = 0, . . . , 3) corresponds to situations in which one toxicity is observed in the first cohort of 3 patients enrolled at the current dose and t toxicities are observed in the second cohort of patients enrolled at that dose.

Modifications to the Traditional Design

A fundamental conflict in Phase I trial design exists between escalating too quickly, resulting in the potential exposure of patients to excessive toxicity, and escalating too slowly, resulting in the treatment of patients at doses too low to be efficacious.[19] A major criticism of the traditional Phase I design is that the potential exists for many patients to be treated at subtherapeutic dose levels. In addition, the length of time these studies often take can inhibit the ability to rapidly bring new agents to subsequent Phase II and Phase III studies. Several variations to the traditional design have been developed in order to reduce the number of patients treated at doses below the biologically active level and to improve on the precision of the MTD definition. Some of the most commonly used types of modified traditional designs include those proposed by Storer[24] and by Simon and colleagues.[18]

The Storer BD design uses a two-stage approach.[24] In the first stage, only a single patient is entered at each dose level. Dose escalation continues with one-patient cohorts until a DLT is observed. Accrual to the second stage then begins at one lower dose level and follows the traditional (three-patient cohort) design. Such a scheme allows fewer patients to be treated at dose levels less likely to be efficacious. Storer also proposed defining the MTD by fitting the first-course toxicity data to a logistic dose-toxicity curve and letting the MTD be defined as the dose level associated with a target DLT rate (e.g., 20% to 30%).[24] This allows for a more precise MTD definition.

Simon described three types of accelerated titration designs that were modifications of the traditional design (referred to by Simon as Design 1).[18] The Simon Design 2 is similar to the Storer design in that it uses single-patient cohorts during the initial stage, but the switch to the second stage (the traditional design) occurs when either the first instance of first course DLT is observed or if two patients exhibit grade 2 toxicity, as defined by the CTCAE, during their first course of treatment.[18] The Simon Design 3 mimics Design 2, except for the incorporation of more rapid dose escalation by using double-dose steps during the single-patient cohort stage. Finally, the Simon Design 4 is similar to Design 3, except switching to the second (three-patient cohort) stage may occur when either the first instance of a DLT occurs or the second instance of grade 2 toxicity is observed in *any* course of treatment. The three Simon

accelerated titration designs also allow for intrapatient dose escalation, permitting escalation for an individual patient if toxicity during their previous course was less than grade 2 as defined by the CTCAE and did not result in a DLT. Accelerated titration designs have become very popular, as they can dramatically reduce the number of patients required, shorten the duration of the trial, and provide a great deal of information about cumulative toxicity, interpatient variability, and steepness of the dose-toxicity curve.[25] Most importantly, they provide all patients a maximum opportunity to be treated at a therapeutic dose. In reviewing several accelerated titration–design Phase I trials, it was identified that the advantages of its use from a prospective perspective were a minimal amount of patients needed to reach the MTD, a lower percentage of patients treated at potentially subtherapeutic doses or with an ineffective agent, and cost containment.[26-30] However, accelerated titration designs did not expedite the speed of completion of studies overall, relative to traditional designs when compared to matching studies done in high-throughput Phase I centers.

Cytotoxic versus Targeted Design

One of the assumptions inherent in the traditional Phase I design is that both toxicity and clinical benefit will increase as the dose of an agent increases. For cytotoxic therapeutic agents, this assumption usually holds true. Recently, however, several agents have been developed that target specific tumor characteristics, such as receptor function, and these agents may not follow the standard efficacy/toxicity model. Specifically, targeted agents may demonstrate a plateau on the dose-efficacy curve, meaning higher doses will not improve clinical benefit. In addition, toxicity occurring with the use of these agents, if it occurs at all, may not necessarily increase as the dose increases. For drugs of this type, determining the MTD may not be feasible or useful. For targeted agents that do not produce immediate or consistent drug-related toxicity, three categories of alternative endpoints have been considered: (1) measuring inhibition of a target, (2) plasma drug levels that are biologically relevant, and (3) surrogate markers of biologic activity in nontumoral tissues.[31]

Several Phase I trial designs have been developed for studies examining targeted, noncytotoxic agents.[32-34] Hunsberger and colleagues proposed several designs based on the assumption that there is a binary (positive or negative) response that is measured in each patient after treatment with an agent; this response indicates whether or not the desired effect has been achieved.[32] The simplest of these designs mimics the traditional 3+3 design but adapts it to examine response rather than toxicity. The goal of this design is to recommend the lowest dose meeting a predefined level of activity (response) for further testing. Dose escalation occurs when a predefined number of responses are not observed. Dose de-escalation will occur if the predefined level of activity has been exceeded.

Pharmacokinetically Guided Dose-Escalation Method (PGDE)

The pharmacokinetically guided dose-escalation (PGDE) method of clinical trial design was proposed by Collins and associates as a more informative and efficient alternative to the traditional design.[19,35] The authors retrospectively analyzed the results of several Phase I studies of chemotherapeutic agents and demonstrated that observed toxicity was not a function of the dose administered to the patient, but rather was a function of the area under the curve (AUC) of plasma drug concentration measured over time of exposure. The PGDE Phase I clinical trial design targets the AUC associated with the mouse LD_{10}. Patients are treated at one-tenth of the mouse LD_{10}, as in the traditional method, but escalation to the next dose and subsequent doses is based on the distance of the observed AUC in humans to the target mouse LD_{10} AUC. The retrospective analysis performed by Collins and colleagues indicated that the sample size of Phase I clinical trials could be reduced by as much as 50% by using the PGDE over the traditional design.[19] Although several studies have reported success with the PGDE design, it is still not widely used in the drug development community.[36] One reason for the lack of use is the presence of large interpatient variability in AUC for the same administered dose.[25] For some drugs (e.g., antimetabolites and vinca alkaloids), toxicity is a function of exposure time above a threshold rather than AUC, and the use of a PGDE design is not justified.[37] Finally, the requirement of real-time pharmacokinetic monitoring inherent in the PGDE design has been considered a limitation to its use.[36,38] Pharmacokinetic correlative studies, however, have become standard measurements in almost all oncology Phase I trials as they help to better understand Phase I trial outcomes.

Continual Reassessment Method (CRM)

O'Quigley and co-workers proposed the Continual Reassessment Method (CRM) as an alternative Phase I study design. This Phase I design uses formal statistical methods of dose-toxicity modeling to guide dose escalation.[39] The CRM is considered superior by many because it allows the use of toxicity information gained at earlier time points of the study to assign subsequent doses. The CRM design is considered less likely to treat patients at toxic doses and more likely to

treat patients at doses considered efficacious.[40] The CRM, as originally designed, works by fitting a dose-toxicity curve to the available toxicity data and assigns subsequent patients to the dose most likely to be associated with a predefined target toxicity level. Therefore, the MTD is defined as the dose estimated to produce a desired predefined toxicity rate. The estimated dose-toxicity curve is refitted after the outcome of each individual patient is determined, and the next patient is assigned the dose estimated to be nearest the MTD based on the new data.[40] Because of its complexity, involvement of a capable statistician is necessary in the design and execution of a CRM-designed clinical trial.

Statistical Considerations of Phase I Studies

There are many designs available to estimate the MTD, as discussed earlier. Two of the main design types used in practice are either algorithmic in nature (e.g., the previously described 3+3 design) or model-based designs (i.e., designs based on a statistical model). The purpose of the 3+3 design is not to produce accurate estimates of the probability of toxicity at a given dose but to quickly identify a dose level that does not exhibit too much toxicity. An alternative to algorithmic approaches such as the 3+3 design, and one more amenable to the goal of precisely estimating (i.e., estimating with more certainty) the MTD, is model-based methods. The conceptual framework for most model-based Phase I designs is Bayesian. Bayesian designs treat the probability that a patient will experience toxicity at a given dose as a quantity about which the investigator has some degree of uncertainty. Moreover, this uncertainty is quantified via probability. The Bayesian framework provides a means by which one can learn about the toxicity rates at the different doses and naturally make decisions based on the data observed in a sequential manner.

Using these model-based designs requires that the investigator *explicitly* specify a target probability of toxicity. The target probability of toxicity represents the rate of toxicity acceptable to the investigator (the 3+3 design has an *implicit* target rate of toxicity of approximately 17%). For compounds associated with very severe life-threatening toxicities, the target probability may be set by the investigator at 0.10 (i.e., 10%), whereas for other compounds with more mild toxicities it may be acceptable to set the target probability of toxicity at 0.35. As with algorithmic designs, patients are sequentially enrolled into the trial in cohorts of patients. After each cohort of patients has been evaluated for toxicity, the decision to escalate, stay, or de-escalate from the current dose is based on the dose that has the expected probability of toxicity closest to the target toxicity.

An important advantage of model-based Phase I designs is that they allow one to combine information from patients treated at different dose levels, that is, to "borrow strength," in order to more reliably predict what may occur at a particular dose given to a future patient. A second advantage is the ability to adjust the target probability of toxicity to match the characteristics of the compound under investigation. A third advantage of model-based methods is that, unlike the 3+3 design, the cohort size is not limited to 3 patients and, more importantly, a variable cohort size may be used. Although one could argue that algorithmic designs can also use alternative cohort sizes, the complication associated with changing the cohort size when using "X+X" algorithmic approaches (e.g., 2+2, 4+4, 5+5) is that the implicit targeted rate toxicity changes with the size of the cohort. We should note that there are other algorithmic designs which do not tie the implicit target toxicity rate to the cohort size but these methods are very rarely used and tend to place too many patients on doses that are too toxic (reviewed in Ivanova and colleagues[41]).

Although model-based designs have been available since the early 1990s, these methods have not gained as wide an audience as biostatisticians would like. This is because it can be difficult to explain these methods to nonstatisticians, and the methods are difficult to implement.[42] These difficulties are being addressed by making computer code available to investigators and by providing innovative designs that target endpoints other than the typical endpoint in a classical implementation of a Phase I oncology design.

Pharmacodynamic Markers in Phase I Studies: Tissue Analysis

Overview of Pharmacodynamic Markers in Tissues

In recent years, there has been significant progress in the development of drug-targeted therapies, particularly those that target receptor tyrosine kinases (RTKs).[43,44] The emergence of molecularly targeted agents against numerous targets offers potentially greater anticancer efficacy with fewer side effects. Despite these recent advances, assessing the effects of these agents individually or in combination, or combined with conventional therapies, has created significant challenges for basic scientists and clinical investigators to effectively integrate molecular targeted therapies into clinical practice.[45] Because the number of possible drug-target combinations is enormous, better strategies are needed to understand the pharmacodynamic effects of

investigational agents in tumors.[46] One of the most informative approaches is to implement correlative tissue-based analyses in clinical studies.[47] This section discusses the development of reliable assays for quantifying pharmacodynamic effects in tissues, the effects of different agents on various markers and their correlation with clinical outcome, and issues that pose challenges for incorporating tumor tissue analysis into clinical trials.

Quantitative Analysis of Pharmacodynamic Markers in Tissues

Investigators typically rely on immunohistochemistry (IHC) assays to measure the pharmacodynamic effects of molecular targeted therapies in tissues. The majority of these studies use chromogenic or immunoperoxidase staining, which are semiquantitative and have other limitations.[48] In contrast, immunofluorescence (IF) detection methods can provide simultaneous labeling of multiple proteins in one sample and a quantitative assessment using a continuous scale.[49] Recent research efforts have focused on the development of IF-based assays to quantify protein expression patterns and apoptosis in tissues for Phase I studies (Figure 48-2).[50,51] Initially, this work focused on developing a method to detect apoptosis in endothelial cells, which requires three fluorochromes to visualize the total cell nuclei, endothelial cells, and terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL)-positive cells.[52] Hence, multiple labeling techniques can facilitate visualization of specific cell types by eye as a result of colocalization of different fluorochromes (see Figure 48-2). However, manual quantification is limited to enumerating "positive" and "negative" cells in random microscopic fields using a categorical score and may not be able to detect subtle but significant changes.[53]

Various platform technologies have been developed to facilitate quantitative in situ assessment of protein expression.[54] Most of these systems are designed for standard IHC assays using chromogenic substrates. Measuring the

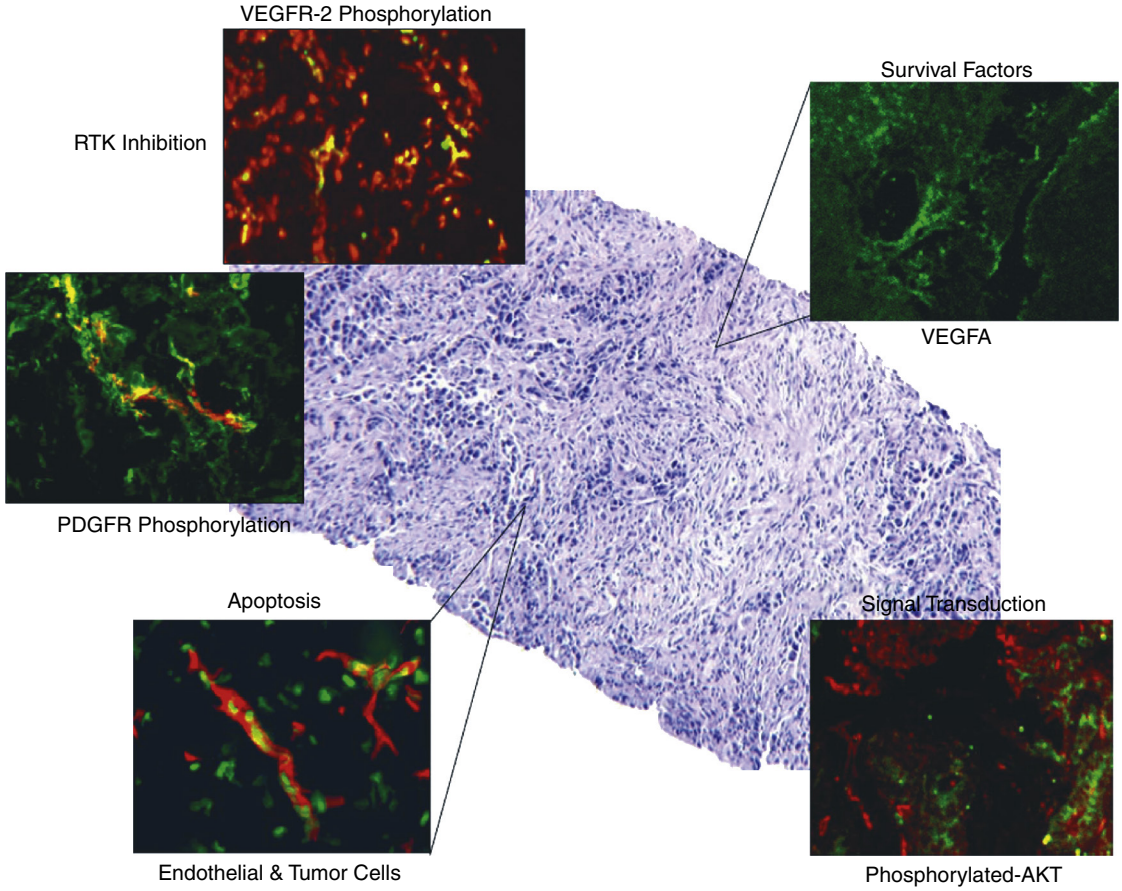


Figure 48-2 Pharmacodynamic analysis of molecular targeted therapies in tumor tissues. Correlative tissue studies may help determine the pharmacodynamic effects of targeted therapies on receptor tyrosine kinase phosphorylation, growth factors, signal transduction, and apoptosis in Phase I studies. Immunofluorescence detection permits the analysis of biomarkers in specific cell types, such as phosphorylation of PDGFR-β in endothelial cells. Measuring endpoints that include target or pathway inhibition linked to apoptosis may provide better evidence of the biological effects of the drug in the tumor and correlation with clinical outcome. (*Red*, endothelium; *green*, protein expression or terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL); *yellow*, colocalization of endothelium and protein or TUNEL.)

pharmacodynamic effects of molecular targeted therapies requires the ability to detect specific cell types, such as endothelial cells, and quantify their protein expression patterns. One platform technology capable of quantifying multiple fluorochromes in fixed tissue specimens is the laser scanning cytometer (LSC). The LSC platform is an automated analysis system described as a cross between a flow and a static image cytometer. Lasers are used to simultaneously excite different fluorochromes in cellular specimens that emit discrete wavelengths detected by a set of photomultiplier tubes. Together these features permit the ability to generate high-content stoichiometric data on heterogeneous populations of large numbers of cells. Thus, the LSC is used much like a flow cytometer to obtain multicolor immunofluorescence intensity information on fixed specimens.

Several Phase I studies have incorporated LSC-mediated analysis to determine drug-target interactions, effects on downstream signaling pathways, and rates of apoptosis in skin and tumor tissues.[49-51,55] Because the LSC is a platform technology, many different applications can be developed to exploit its inherent capabilities. Research efforts have been focused on developing specific tissue-based applications using LSC technology in an attempt to standardize the methodology for consistent data generation that can be compared between different tissue specimens and molecular targeted therapies. Although LSC-mediated data acquisition is automated, the process requires a systematic interactive approach to maintain high quality-control standards and ensure consistent data generation (Figure 48-3). Pharmacodynamic data generated using a process to analyze markers in entire tumor tissue cross sections has consistently provided biological evidence of the effects of targeted therapies and correlation with clinical outcome.[49,56,57]

Pharmacodynamic Analysis of Receptor Tyrosine Kinase Targeted Therapies

Aberrant expression of cell-surface RTKs, such as epidermal growth factor receptor (EGFR), plays a pivotal role in the progression of cancer.[58] Drugs that target RTKs are designed to block the intrinsic enzymatic activity that catalyzes the transfer of the gamma-phosphate of ATP to tyrosine residues in protein substrates.[59] Inhibiting phosphorylation of these tyrosine residues prevents downstream signaling events, which affect cellular function (e.g., proliferation, differentiation, migration, or apoptosis).[60] Thus, the ability to measure phosphorylation status and signal-transduction pathways has become an important pharmacodynamic endpoint in clinical studies.

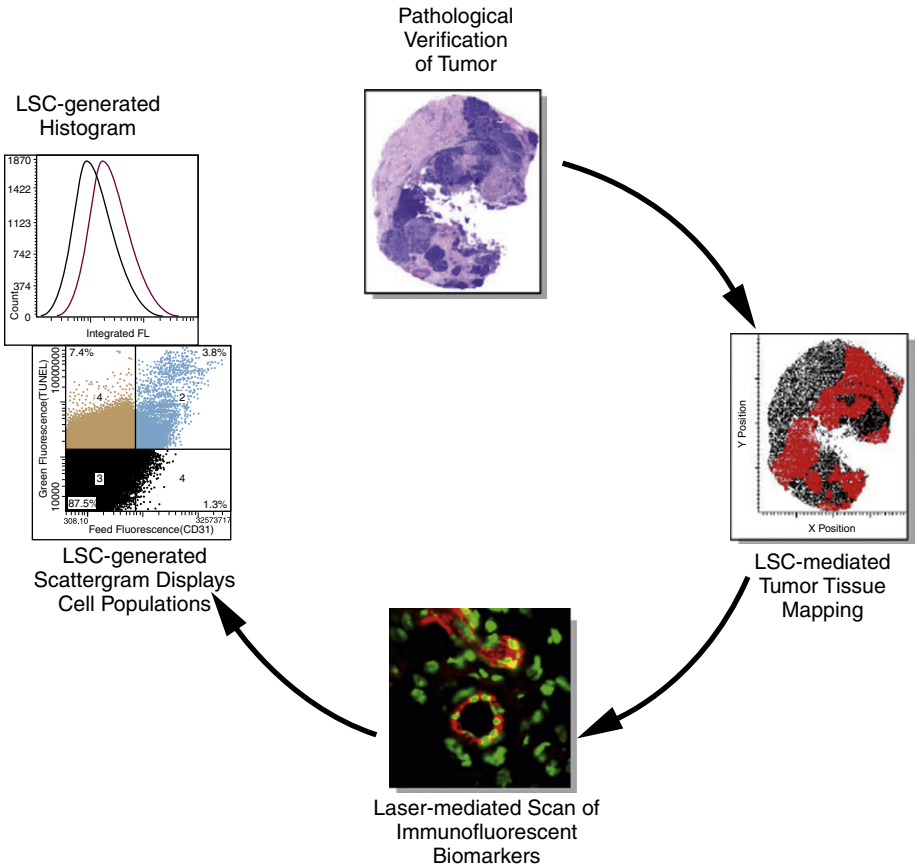


Figure 48-3 Quantitative analysis of pharmacodynamic effects in tissues using LSC technology. Pathological verification of biopsy samples is essential for mapping tumor regions and excluding normal and necrotic regions from the analysis. Lasers detect individual cells within the mapped region of interest based on immunofluorescence staining. LSC-generated scattergrams display the percentage of cell populations based on user-defined gating using controls, e.g., apoptotic endothelial cells. Alternatively, protein expression levels, such as phosphorylated VEGF receptor-2, measured by mean fluorescent intensity may be determined as shown in the histogram. *(Immunofluorescent image appears with permission of Eaton Publishing, Westborough, MA 01581, USA; Cover,* BioTechniques, *Vol. 28, No. 6 (June 2000)).*

Table 48-2 Recent Successful Phase I Trials

Drug	Patients	Response Rate	Stable Disease	Disease Control
Erivedge[65]	33	55%	33%	88%
Vemurafenib[66]	16	69%	19%	88%
Crizotinib[67]	10	30%	40%	70%

Gefitinib

Gefitinib (Iressa, ZD1839) was the first in a new class of small, molecular targeted therapies against EGFR to gain market approval (based on two Phase II studies) for non–small-cell lung cancer.[61,62] Although the Phase II studies did not incorporate correlative tissue studies, it was demonstrated in two different Phase I studies of gefitinib that pharmacodynamic endpoints can be measured in both tumor and skin tissues. In a metastatic colorectal cancer trial, total EGFR levels; phosphorylation of EGFR, AKT, and ERK; p27 levels; beta-catenin expression; and apoptosis were assayed before and after treatment in tumor biopsies, with interesting results in only a small number of patients. In another Phase I study of gefitinib in metastatic breast cancer, comparison of pre- and posttreatment ERBB2 and EGFR values was not statistically significant between the subgroups of patients regarding responsiveness to treatment.[63]

Serial skin biopsies have been analyzed as potential surrogate tissues for monitoring the biologic effects of molecular targeted therapies. A Phase I study of gefitinib in advanced solid malignancies incorporated skin, but not tumor, biopsies to determine the effects on EGFR signaling.[64] Levels of phosphorylated-EGFR expression were completely inhibited; however, no changes in total EGFR expression were observed after treatment. Other downstream markers in the EGFR network were affected by ZD1839, including phosphorylated-Ras-mitogen-activated protein kinase (MAPK) and STAT3, Ki67, p27(kip1), and apoptosis (Table 48-2).[65-67] Although significant changes were observed in almost all of the markers when comparing pre- and posttreatment skin biopsies in small numbers of patients, none of the changes correlated with dose or clinical response.

Pharmacodynamic Analysis of Signal Transduction Inhibitors and Other Targets

Vemurafenib

Vemurafenib (PLX4032, Zalboraf) is an ATP-dependent serine/threonine kinase inhibitor approved for use in patients with stage IV melanoma with BRAF(V600E) mutations. Forty percent to 60% of melanomas, and 7% to 8% of all cancers, carry an activating mutation in *B-RAF*. Ninety percent of reported *BRAF* mutations result in a substitution of glutamic acid for valine at amino acid 600—the V^{600} mutation. This BRAF mutation constitutively activates BRAF and downstream signal transduction in the MAP kinase pathway. Preclinical studies showed that vemurafenib inhibits the kinase activity of BRAF with the V^{600} mutation at low nanomolar concentrations, abrogates signaling through the MAP kinase pathway, and blocks proliferation of cells carrying BRAF with the V^{600} mutation in vitro at high nanomolar concentrations.

In the Phase I clinical trial, the extent of pathway inhibition and tumor responses correlated with higher plasma drug exposures. Patients with vemurafenib plasma exposures (AUC_{0-24}) less than 300 mM hr experienced no measurable tumor responses, whereas 24 of 32 patients treated at the MTD/RP2D of 960 mg BID had PRs or CRs, where the AUC_{0-24} was 1741 μM hr. Paired biopsies were taken from selected patients, at baseline and after 14 days of exposure. Decreases in cytoplasmic, though not nuclear, pERK correlated well with tumor response. Greater than 80% inhibition of cytoplasmic ERK phosphorylation was observed in responding patients, which suggests that near-total inhibition of BRAF signaling is required for clinical benefit.[68]

Aflibercept (VEGF-trap, Eylea) is a recombinant protein consisting of segments of the extracellular domains of human vascular endothelial growth factor receptors 1 (VEGFR1) and 2 (VEGFR2) fused to the constant region (Fc) of human IgG1. Aflibercept functions as a soluble decoy receptor, binding to blood vascular endothelial growth factors (VEGFs) and preventing VEGFs binding to the VEGFR-1, -2 receptors. Aflibercept is approved for use in age-related macular degeneration and in combination with chemotherapy for colorectal cancer. In Phase I clinical trials in cancer patients, the saturation of aflibercept by circulating VEGF was used to determine the appropriate dose for Phase II/III trials. Preclinical studies demonstrated a requirement for free aflibercept to exceed the VEGF-aflibercept complexes for antitumor activity. At doses of 2 mg/kg[69] and 800 mg,[70] there was no further increase in complex formation, and free drug levels remained in excess of bound aflibercept levels. These concentrations correlated with DCE-MRI effects on tumor perfusion (see later discussion), immunologic evidence of VEGF binding, and clinical outcome, thus allowing selection of a biologically active dose on a rational basis.

Recent Therapeutic Successes with Phase I Trials

Although the traditional role for Phase I studies has been the evaluation of toxicity of the MTD and RP2D, with

efficacy evaluated in expansion cohorts and Phase II trials, recent studies with enriched populations for specific targets have shown significant responses, followed by successful randomized Phase III trials and FDA approval, although the approval occasionally occurred even before or in the absence of the Phase III trial (see Table 48-2). Erivedge (GDC0449) received FDA approval after remarkable results from the Phase I trial, where responses were seen in 18 (55%) of 33 patients with locally advanced or metastatic basal cell carcinomas, with an additional 11 (33%) patients achieving stable disease; progression was seen in only 4 patients.[65] In the Phase I trial of vemurafenib (PLX4032), in patients with melanoma harboring the BRAF V600E mutation, complete or partial responses were observed in 11 (69%) of the 16 patients treated in the dose-escalation phase and 26 (81%) of the 32 patients treated at the extension phase with the recommended Phase II doses.[66] Vemurafenib was subsequently approved after the results of a randomized Phase III trial showing improved overall survival compared to dacarbazine in patients with BRAF V600E mutated in previously untreated patients with metastatic melanoma.[71] In the Phase I trial of crizotinib (PF-20341066), there were 3 responders and 4 patients with stable disease, including 3 patients with tumor reduction of 20% and one with stable disease for 28 weeks, among the 10 patients with ALK rearranged non–small-cell lung cancer.[67] The promising results were confirmed in the Phase II study, where 47 (57%) of 82 patients with ALK rearrangement achieved partial response, and 27 (33%) had stable disease, mostly by tumor shrinkage that did not meet criteria for partial response.[72] These highly successful drugs may change the paradigm in Phase I studies, bypassing Phase II studies to move directly into randomized Phase III studies in an attempt to allow rapid access to drugs with the potential to significantly improve survival in selected patient populations.

Challenges and Perspectives

There are many challenges involved in successfully incorporating tissue analysis in the design of a clinical study. Acquiring the tissue alone requires the commitment of the sponsor, scientists, oncologists, interventional radiologists, committees, and patients. Standardization of tumor sampling, tissue procurement, and storage procedures is critical to ensure that quality tumor tissue is being evaluated. A lack of quantitative standardization among different assays may lead to unintentional interpretation and variability among laboratories. Other issues that may affect interpretation of pharmacodynamic data are intra- and intertumor heterogeneity, tissue microenvironment (skin versus tumor), compensatory mechanisms, and timing of biopsies after initiation of therapy and after the last dose. It is worth emphasizing that few studies have attempted to link target or pathway inhibition with tumor-cell apoptosis. It is possible that some agents may demonstrate transient target inhibition, but fail to induce apoptosis.[49] Thus, measuring pharmacodynamic endpoints that include target or pathway inhibition linked to cellular fate, such as apoptosis, may provide better evidence of the biological effects of the drug in the tumor.

Pharmacodynamic analysis of tumor tissues can provide direct proof of whether an investigational agent affected its intended target and downstream consequences on signal transduction and apoptosis; however, such studies are limited. Recent studies have demonstrated that skin may serve as a surrogate tissue to confirm drug-target inhibition, signal transduction, and kinetics in clinical studies. However, analysis of biomarkers in tumor tissues may better represent the biological effects of a targeted therapy, as tumor cells often respond differently compared to normal cells. More quantitative studies are needed to identify reliable biomarkers and the correlation between the effects in skin, tumor, and clinical outcome. Another promising surrogate source that could potentially be used to assess the effects of targeted agents is the circulating tumor cell or endothelial cell. These cells may better represent the tumor microenvironment and are now being routinely isolated for a variety of applications.[73] Ongoing research efforts are aimed at developing assays to analyze the pharmacodynamic effects of drugs on circulating tumor and endothelial cells. Furthermore, pharmacodynamic studies in tumor tissue may also identify the genomic and proteomic profile of the population with the greatest chance to benefit from treatment. For example, the therapeutic activity of trastuzumab (Herceptin) would likely have been missed if patients had not been preselected based on their HER2 status.

Clearly, there is a need for better strategies to assess the effects of molecular targeted therapies early in clinical development. For example, in a Phase I trial of bevacizumab, no objective responses were observed out of 25 patients.[74] Not until a series of randomized Phase II and III trials over a period of more than 5 years was the clinical activity of bevacizumab established. However, it is generally not practical to perform large randomized trials for drugs without evidence of biological activity early in their development, and therefore, many promising drugs may not be developed. Given the large number of targeted therapies entering clinical testing, it is crucial that Phase I studies incorporate correlative endpoints to determine biological activity and optimal dosing and scheduling for Phase II and III trials. Ultimately, clinical development of targeted therapies would benefit if the recommended dose was identified early and actually known to inhibit the target for which it was designed.

Imaging Techniques in Phase I Studies

A variety of imaging techniques can play an important role in Phase I studies of anticancer drugs when used as an objectively measured indicator of a biological/pathobiological process or pharmacologic response to treatment (i.e., as a biomarker[75]). Imaging biomarkers can be used to determine if the drug is hitting the target and if it has the anticipated biological activity, and they can also provide an early indication of whether or not the new agent has clinical activity.[76] The information provided by imaging biomarkers, taken together with information from molecular biomarkers and clinical pharmacology, provides the input required to determine how aggressively to pursue the development of a particular drug or a backup drug for a given target. In addition, imaging biomarkers can assist in the selection of the dose and/or schedule for Phase II studies.[77]

The ability to detect a labeled drug at one thousandfold lower concentrations than needed to produce pharmacodynamic effects makes nuclear medicine the modality best suited for determining if the drug is hitting the target.[78] A number of imaging modalities can be used to determine if the drug has the anticipated biological or antitumor activity. The more commonly used methods are dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and [^{18}F]fluoro-2-deoxyglucose (FDG) positron emission tomography (PET). DCE-MRI, which employs a commonly used contrast agent (gadopentetate dimeglumine), has been implemented in several Phase I studies to quantify the effects of antivascular agents on the tumor blood supply within hours to days after the start of treatment.[79] Further research will be needed to determine if it is a suitable marker for predicting clinical activity.

FDG-PET uses an ^{18}F-labeled glucose analog (FDG) that is transported into cells by GLUT-1 and GLUT-3 and phosphorylated by hexokinase. Because FDG-6-P is a poor substrate for glucose-6-phosphatase, there is little dephosphorylation, and the radioactivity is trapped in the cell.[80-82] Glucose metabolism is quantified as the activity in the tumor, normally restricted to the region of highest activity, relative to the amount of activity injected and patient's body weight, the so-called Standardized Uptake Value (SUV). The potential for FDG-PET to assess drug-induced biological effects before a change in tumor size was illustrated clearly in patients with advanced gastrointestinal stromal tumors treated with imatinib mesylate.[83] Tumor FDG activity decreased markedly from baseline as early as 24 hours after a single dose of imatinib in all patients demonstrating a response by computed tomography (CT) or MRI weeks later. Conversely, increased tumor FDG activity, activity at new sites, or both were seen in all patients with disease progression evident at a later date by conventional means. In patients with progressive disease after treatment with imatinib, FDG-PET metabolic response, defined as SUV decrease or increase by 25% from baseline at 4 weeks, provided an early prediction of response to sunitinib.[84] Although a variety of treatment regimens result in reduced FDG activity following the first cycle of therapy, after macrophage activity (which can result in increased FDG uptake) has subsided, yet before response is evaluable by standard methods,[80] such dramatic effects are not generally observed so early after treatment. Nonetheless, FDG-PET shows considerable promise to provide an indication of decreased tumor viability earlier than conventional methods and may provide a valuable downstream biomarker for biologic activity in Phase I trials.

Although it is not reasonable to expect clinical efficacy in the advanced-stage patients entered into Phase I trials and assessment of clinical response is not a primary focus of Phase I trials, any indication that the drug/target affects tumor growth is beneficial. Typically, tumor burden is assessed using either CT or MRI data. The method most commonly used to assess clinical effect is based on Response Evaluation Criteria in Solid Tumors (RECIST), which was put forth in 2000 as a simpler way to measure the response of tumors to experimental treatments.[85] The recently updated criteria (RECIST 1.1) include important changes such as the inclusion of cystic bone lesions with identifiable soft tissue components and progressing previously irradiated lesions as measurable disease, definition of measurable lymph nodes as those with shortest axis at least 1.5 cm, and progressive disease by PET scan defined as the presence of positive PET in a previously negative area.[86] It should be noted that, in practice, RECIST are generally modified to address some of the concerns raised by the International Cancer Imaging Society (ICIS) regarding the strengths and weaknesses of using the RECIST criteria and what other issues should potentially be added to a response criterion.[87] Nonetheless, even with these changes, concerns remain regarding RECIST, especially in the context of early-phase trials.[88] One point of particular concern is whether the single longest tumor dimension, determined in an axial plane, accurately represents changes in tumor burden, because most tumors grow and regress irregularly.[89] Another concern is how relevant the categorical response assessments (complete response, partial response, stable disease, and progressive disease), which were originally based on the error in oncologists' physical measurements of solid spheres arranged in random size order on a soft mattress and covered with a layer of foam rubber, are in the context of early-phase trials.[90,91] It seems an alternative model, where response is considered a continuous variable, the change in tumor size (estimated as the single longest dimension, the cross product of the longest dimension and the perpendicular longest dimension, or volume) after treatment,[92] would be much more useful for evaluating clinical effect in Phase I trials. Some of the limitations from the RECIST criteria, including the evaluation of targeted therapies that frequently cause disease stabilization instead of objective response, may be addressed with the proposed PET

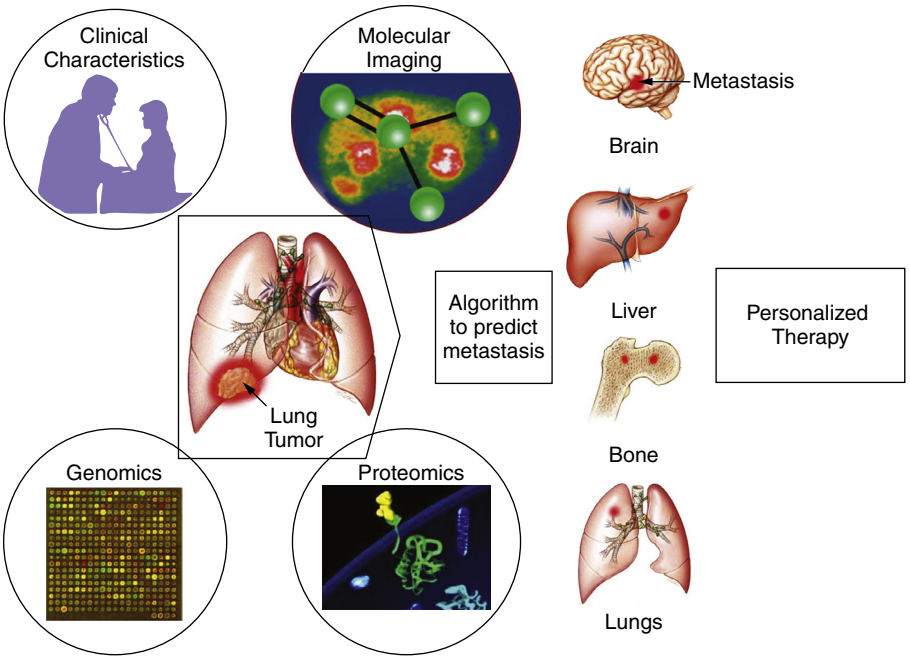


Figure 48-4 An algorithm comprising relevant proteomic, clinical and imaging factors plus genomic factors all indicating a poor prognosis (metastasis) and best choice of molecular-targeted chemotherapy.

response criteria in solid tumors (PERCIST), which classifies the metabolic response into complete (CMR), partial (PMR), stable (SMD), or progressive (PMR) based on complete resolution of FDG uptake in the measurable target lesions, reduction of at least 30% of the FDG uptake, changes from 29% decrease to 30% increase in FDG uptake, or increase of at least 30% in the FDG uptake, respectively.[93]

Conclusion

Cancer is and will be a major cause of death and morbidity in the United States and worldwide. Despite significant improvements in diagnosis, surgical techniques, general patient care, and local and systemic therapies, most deaths from cancer are still due to metastases that are resistant to conventional treatment. Novel therapeutic approaches are critically needed if we are to improve patient outcome. Phase I studies are the critical link in targeting cancer, because they represent the first translation of years of laboratory/preclinical studies to the patient.

As drug development has evolved to a more tumor-targeted or tumor-specific focus, so has the evolution of Phase I trials moved from the more generic, mathematical modeling to a more rational design. In addition, it is increasingly being recognized that incorporation of select endpoints relative to patient eligibility in Phase I trials is needed to more effectively and efficiently develop drugs clinically. Although the classification of most cancers is still based in large part on tissue type, tumor size, nodal status, and metastatic sites, there has been a rapid progress in the molecular characterization of solid tumors. Several Phase I designs are incorporating these tools, not so much as response predictors, but to help determine feasibility and to develop diagnostic/predictive tools for future clinical use. The hope is that a more personalized approach to clinical care will increase the efficacy of treatment, while decreasing its toxicity and cost. The end result is the development of Phase I trials aimed not only at defining dose and safety, but also at assisting in target validation while increasing the probability of benefit through the use of enriched populations (Figure 48-4). Recent studies have shown that effective drugs can be more expeditiously approved if used in a molecularly defined patient population, with a rapid transition from Phase I studies to proof-of-concept Phase II and randomized Phase III trials, with more rapid introduction into clinical practice.

Key References

1. Horstmann E, McCabe MS, Grochow L, et al. Risks and benefits of phase 1 oncology trials, 1991 through 2002. *N Engl J Med*. 2005;352:895-904.
2. Trump DL. Hospice benefits and phase I cancer trials. *Ann Intern Med*. 2004;140:70-71; author reply 71.
3. Travis K. For phase I studies, ethical and practical concerns abound. *J Natl Cancer Inst*. 2004;96:1354-1355.
4. Agrawal M, Emanuel EJ. Ethics of phase 1 oncology studies: reexamining the arguments and data. *JAMA*. 2003;290:1075-1082.
5. Roberts D, Lockley W. Radiosynthesis—a vital role supporting drug development? *Drug Discov World*. 2004;5:59-62.
6. Rudin M, Weissleder R. Molecular imaging in drug discovery and development. *Nat Rev*. 2003;2:123-131.

7. Kelloff GJ, Krohn KA, Larson SM, et al. The progress and promise of molecular imaging probes in oncologic drug development. *Clin Cancer Res*. 2005;11:7967-7985.
8. Rahman A, White RM. Cytotoxic anticancer agents and renal impairment study: the challenge remains. *J Clin Oncol*. 2006;24:533-536.
9. Takimoto CH, Mita AC. Design, conduct, and interpretation of organ impairment studies in oncology patients. *J Clin Oncol*. 2006;24:3509-3510; author reply 3510-3501.
10. Ramanathan RK, Remick SC, Mulkerin D, et al. P-5331: a phase I pharmacokinetic (PK) study of STI571 in patients (pts) with advanced malignancies and varying degrees of liver dysfunction (LD). *Proc Am Soc Clin Oncol*. 2003:22; abstr 502.
11. Remick SC, Ramanathan RK, Mulkerin D, et al. P-5340: a phase I pharmacokinetic study of STI-571 in patients (pts) with advanced malignancies and varying degrees of renal dysfunction. *Proc Am Soc Clin Oncol*. 2003:22; abstr 503.
12. Doroshow JH, Synold TW, Gandara D, et al. Pharmacology of oxaliplatin in solid tumor patients with hepatic dysfunction: a preliminary report of the National Cancer Institute Organ Dysfunction Working Group. *Semin Oncol*. 2003;30(4 suppl 15):14-19.
13. Takimoto CH, Remick SC, Sharma S, et al. Dose-escalating and pharmacological study of oxaliplatin in adult cancer patients with impaired renal function: a National Cancer Institute Organ Dysfunction Working Group Study. *J Clin Oncol*. 2003;21:2664-2672.
14. Takimoto CH, Remick SC, Sharma S, et al. Administration of oxaliplatin to patients with renal dysfunction: a preliminary report of the national cancer institute organ dysfunction working group. *Semin Oncol*. 2003;30(4 suppl 15):20-25.
15. LoRusso PM, Venkatakrishnan K, Ramanathan RK, et al. Pharmacokinetics and safety of bortezomib in patients with advanced malignancies and varying degrees of liver dysfunction: phase I NCI Organ Dysfunction Working Group Study NCI-6432. *Clin Cancer Res*. 2012;18:2954-2963.
16. Cavero I, Crumb W. The use of electrocardiograms in clinical trials: a public discussion of the proposed ICH E14 regulatory guidance April 11-12, 2005, Bethesda, MD, USA. *Expert Opin Drug Saf*. 2005;4:795-799.
17. LoRusso PM, Heath E, Valdivieso M, et al. Phase I evaluation of AZD2171, a highly potent and selective inhibitor of VEGFR signaling, in combination with selected chemotherapy regimens in patients with advanced solid tumors. *J Clin Oncol 2006 ASCO Annu Meeting Proc Part I*. 2006;24:3034 (June 20 Supplement).
19. Collins JM, Zaharko DS, Dedrick RL, Chabner BA. Potential roles for preclinical pharmacology in phase I clinical trials. *Cancer Treatment Rep*. 1986;70:73-80.
20. Omura GA. Modified Fibonacci search. *J Clin Oncol*. 2003;21:3177.
22 Zhao L, Lee J, Mody R, Braun TM. The superiority of the time-to-event continual reassessment method to the rolling six design in pediatric oncology Phase I trials. *Clin Trials*. 2011;8:361-369.
23. Skolnik JM, Barrett JS, Jayaraman B, Patel D, Adamson PC. Shortening the timeline of pediatric phase I trials: the rolling six design. *J Clin Oncol*. 2008;26:190-195.
25. Rubinstein L, Simon R. Phase 1 clinical trial design. In: Budman DR, Calvert AH, Rowinsky EK, eds. *Handbook of Anticancer Drug Development*. Baltimore, MD: Lippincott Williams & Wilkins; 2003:297-308.
26. Alousi AM, Parchment R, Boinpally R, et al. Phase I clinical trial of XK469 in patients with chemo-refractory solid tumors. *J Clin Oncol (Meeting Abstracts)*. 2004;22(14 suppl) 2020.
27. Gadgeel SM, Boinpally RR, Heilbrun LK, et al. A phase I clinical trial of spicamycin derivative KRN5500 (NSC 650426) using a phase I accelerated titration "2B" design. *Invest New Drugs*. 2003;21:63-74.
28. Gadgeel SM, Wozniak A, Boinpally RR, et al. Phase I clinical trial of BMS-247550, a derivative of epothilone B, using accelerated titration 2B design. *Clin Cancer Res*. 2005;11:6233-6239.
32. Hunsberger S, Rubinstein LV, Dancey J, Korn EL. Dose escalation trial designs based on a molecularly targeted endpoint. *Stat Med*. 2005;24:2171-2181.
33. Korn EL. Nontoxicity endpoints in phase I trial designs for targeted, noncytotoxic agents. *J Natl Cancer Inst*. 2004;96:977-978.
34. Zhang W, Sargent DJ, Mandrekar S. An adaptive dose-finding design incorporating both toxicity and efficacy. *Stat Med*. 2006;25:2365-2383.
40. Garrett-Mayer E. The continual reassessment method for dose-finding studies: a tutorial. *Clin Trials (Lond)*. 2006;3:57-71.
41. Ivanova A, Montazer-Haghighi A, Mohanty SG, Durham SD. Improved up-and-down designs for phase I trials. *Stat Med*. 2003;22:69-82.
43. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med*. 2005;353:1659-1672.
44. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol*. 2003;21:60-65.
45. Dancey JE, Chen HX. Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat Rev*. 2006;5:649-659.
46. Davis DW, McConkey DJ, Abbruzzese JL, Herbst RS. Surrogate markers in antiangiogenesis clinical trials. *Br J Cancer*. 2003;89:8-14.
48. Rimm DL. What brown cannot do for you. *Nat Biotechnol*. 2006;24:914-916.
49. Davis DW, Takamori R, Raut CP, et al. Pharmacodynamic analysis of target inhibition and endothelial cell death in tumors treated with the vascular endothelial growth factor receptor antagonists SU5416 or SU6668. *Clin Cancer Res*. 2005;11(2 Pt 1):678-689.
50. Davis DW, Shen Y, Mullani NA, et al. Quantitative analysis of biomarkers defines an optimal biological dose for recombinant human endostatin in primary human tumors. *Clin Cancer Res*. 2004;10(1 Pt 1):33-42.
52. Shaheen RM, Davis DW, Liu W, et al. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res*. 1999;59:5412-5416.
53. Berger AJ, Davis DW, Tellez C, et al. Automated quantitative analysis of activator protein-2alpha subcellular expression in melanoma tissue microarrays correlates with survival prediction. *Cancer Res*. 2005;65:11185-11192.
54. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch Pathol Lab Med*. 2006;130:1026-1030.
55. Xiong HQ, Herbst R, Faria SC, et al. A phase I surrogate endpoint study of SU6668 in patients with solid tumors. *Invest New Drugs*. 2004;22:459-466.
56. Davis DW, Buchholz TA, Hess KR, Sahin AA, Valero V, McConkey DJ. Automated quantification of apoptosis after neoadjuvant chemotherapy for breast cancer: early assessment predicts clinical response. *Clin Cancer Res*. 2003;9:955-960.
57. Davis DW, McConkey DJ, Heymach JV, et al. Pharmacodynamic analysis of target receptor tyrosine kinase activity and apoptosis in GIST tumors responding to therapy with SU11248. *Proc Am Soc Clin Oncol*. 2005:23 Abstr 3006.
58. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol*. 2006;33:369-385.
61. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol*. 2003;21:2237-2246.
62. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA*. 2003;290:2149-2158.
63. Gasparini G, Sarmiento R, Amici S, et al. Gefitinib (ZD1839) combined with weekly epirubicin in patients with metastatic breast cancer: a phase I study with biological correlate. *Ann Oncol*. 2005;16:1867-1873.
65. Von Hoff DD, LoRusso PM, Rudin CM, et al. Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med*. 2009;361:1164-1172.
66. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
67. Kwak EL, Camidge DR, Clark J, et al. Clinical activity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. *ASCO Meeting Abstracts*. 2009;27(15S):3509.
68. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467:596-599.
69. Lockhart AC, Rothenberg ML, Dupont J, et al. Phase I study of intravenous vascular endothelial growth factor trap, aflibercept, in patients with advanced solid tumors. *J Clin Oncol*. 2010;28:207-214.
70. Tew WP, Gordon M, Murren J, et al. Phase 1 study of aflibercept administered subcutaneously to patients with advanced solid tumors. *Clin Cancer Res*. 2010;16:358-366.

71. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364:2507-2516.
72. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
73. O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG, Terstappen LW. Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. *Clin Chem*. 2004;50:826-835.
76. Frank R, Hargreaves R. Clinical biomarkers in drug discovery and development. *Nat Rev*. 2003;2:566-580.
77. Collins JM. Functional imaging in phase I studies: decorations or decision making? *J Clin Oncol*. 2003;21:2807-2809.
78. Bergstrom M, Grahnen A, Langstrom B. Positron emission tomography microdosing: a new concept with application in tracer and early clinical drug development. *Eur J Clin Pharmacol*. 2003;59:357-366.
79. Galbraith SM. MR in oncology drug development. *NMR Biomed*. 2006;19:681-689.
80. Kelloff GJ, Hoffman JM, Johnson B, et al. Progress and promise of FDG-PET imaging for cancer patient management and oncologic drug development. *Clin Cancer Res*. 2005;11:2785-2808.
82. de Geus-Oei L-F, van Krieken JH, Aliredjo RP, et al. Biological correlates of FDG uptake in non-small cell lung cancer. *Lung Cancer*. 2007;55:79-87.
84. Prior JO, Montemurro M, Orcurto MV, et al. Early prediction of response to sunitinib after imatinib failure by ^{18}F-fluorodeoxyglucose positron emission tomography in patients with gastrointestinal stromal tumor. *J Clin Oncol*. 2009;27:439-445.
86. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45:228-247.
87. Husband JE, Schwartz LH, Spencer J, et al. Evaluation of the response to treatment of solid tumours—a consensus statement of the International Cancer Imaging Society. *Br J Cancer*. 2004;90:2256-2260.
88. Twombly R. Criticism of tumor response criteria raises trial design questions. *J Natl Cancer Inst*. 2006;98:232-234.
89. Schwartz LH, Mazumdar M, Wang L, et al. Response assessment classification in patients with advanced renal cell carcinoma treated on clinical trials. *Cancer*. 2003;98:1611-1619.
91. Michaelis LC, Ratain MJ. Measuring response in a post-RECIST world: from black and white to shades of grey. *Nat Rev Cancer*. 2006;6:409-414.
93. Verweij J, Therasse P, Eisenhauer E. Cancer clinical trial outcomes: any progress in tumour-size assessment? *Eur J Cancer*. 2009;45:225-227.

This page intentionally left blank

References

1. Horstmann E, McCabe MS, Grochow L, et al. Risks and benefits of phase 1 oncology trials, 1991 through 2002. *N Engl J Med*. 2005;352:895-904.
2. Trump DL. Hospice benefits and phase I cancer trials. *Ann Intern Med*. 2004;140:70-71; author reply 71.
3. Travis K. For phase I studies, ethical and practical concerns abound. *J Natl Cancer Inst*. 2004;96:1354-1355.
4. Agrawal M, Emanuel EJ. Ethics of phase 1 oncology studies: reexamining the arguments and data. *JAMA*. 2003;290:1075-1082.
5. Roberts D, Lockley W. Radiosynthesis—a vital role supporting drug development? *Drug Discov World*. 2004;5:59-62.
6. Rudin M, Weissleder R. Molecular imaging in drug discovery and development. *Nat Rev*. 2003;2:123-131.
7. Kelloff GJ, Krohn KA, Larson SM, et al. The progress and promise of molecular imaging probes in oncologic drug development. *Clin Cancer Res*. 2005;11:7967-7985.
8. Rahman A, White RM. Cytotoxic anticancer agents and renal impairment study: the challenge remains. *J Clin Oncol*. 2006;24:533-536.
9. Takimoto CH, Mita AC. Design, conduct, and interpretation of organ impairment studies in oncology patients. *J Clin Oncol*. 2006;24:3509-3510; author reply 3510-3501.
10. Ramanathan RK, Remick SC, Mulkerin D, et al. P-5331: a phase I pharmacokinetic (PK) study of STI571 in patients (pts) with advanced malignancies and varying degrees of liver dysfunction (LD). *Proc Am Soc Clin Oncol*. 2003;502:22; abstr.
11. Remick SC, Ramanathan RK, Mulkerin D, et al. P-5340: a phase I pharmacokinetic study of STI-571 in patients (pts) with advanced malignancies and varying degrees of renal dysfunction. *Proc Am Soc Clin Oncol*. 2003:22; abstr 503.
12. Doroshow JH, Synold TW, Gandara D, et al. Pharmacology of oxaliplatin in solid tumor patients with hepatic dysfunction: a preliminary report of the National Cancer Institute Organ Dysfunction Working Group. *Semin Oncol*. 2003;30(4 suppl 15):14-19.
13. Takimoto CH, Remick SC, Sharma S, et al. Dose-escalating and pharmacological study of oxaliplatin in adult cancer patients with impaired renal function: a National Cancer Institute Organ Dysfunction Working Group Study. *J Clin Oncol*. 2003;21:2664-2672.
14. Takimoto CH, Remick SC, Sharma S, et al. Administration of oxaliplatin to patients with renal dysfunction: a preliminary report of the national cancer institute organ dysfunction working group. *Semin Oncol*. 2003;30(4 suppl 15):20-25.
15. LoRusso PM, Venkatakrishnan K, Ramanathan RK, et al. Pharmacokinetics and safety of bortezomib in patients with advanced malignancies and varying degrees of liver dysfunction: phase I NCI Organ Dysfunction Working Group Study NCI-6432. *Clin Cancer Res*. 2012;18:2954-2963.
16. Cavero I, Crumb W. The use of electrocardiograms in clinical trials: a public discussion of the proposed ICH E14 regulatory guidance April 11-12, 2005, Bethesda, MD, USA. *Expert Opin Drug Saf*. 2005;4:795-799.
17. LoRusso PM, Heath E, Valdivieso M, et al. Phase I evaluation of AZD2171, a highly potent and selective inhibitor of VEGFR signaling, in combination with selected chemotherapy regimens in patients with advanced solid tumors. *J Clin Oncol 2006 ASCO Annu Meeting Proc Part I*. 2006;24:3034 (June 20 Supplement).
18. Simon R, Freidlin B, Rubinstein L, Arbuck SG, Collins J, Christian MC. Accelerated titration designs for phase I clinical trials in oncology. *J Natl Cancer Inst*. 1997;89:1138-1147.
19. Collins JM, Zaharko DS, Dedrick RL, Chabner BA. Potential roles for preclinical pharmacology in phase I clinical trials. *Cancer Treatment Rep*. 1986;70:73-80.
20. Omura GA. Modified Fibonacci search. *J Clin Oncol*. 2003;21:3177.
21. Schneiderman MA. Mouse to man: statistical problems in bringing a drug to clinical trial. *Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability*. vol IV. Berkeley: University of California Press; 1967;855–866.
22. Zhao L, Lee J, Mody R, Braun TM. The superiority of the time-to-event continual reassessment method to the rolling six design in pediatric oncology Phase I trials. *Clin Trials*. 2011;8:361-369.
23. Skolnik JM, Barrett JS, Jayaraman B, Patel D, Adamson PC. Shortening the timeline of pediatric phase I trials: the rolling six design. *J Clin Oncol*. 2008;26:190-195.
24. Storer BE. Design and analysis of phase I clinical trials. *Biometrics*. 1989;45:925-937.
25. Rubinstein L, Simon R. Phase 1 clinical trial design. In: Budman DR, Calvert AH, Rowinsky EK, eds. *Handbook of Anticancer Drug Development*. Baltimore, MD: Lippincott Williams & Wilkins; 2003:297-308.
26. Alousi AM, Parchment R, Boinpally R, et al. Phase I clinical trial of XK469 in patients with chemo-refractory solid tumors. *J Clin Oncol (Meeting Abstracts)*. 2004;22(14 suppl):2020.
27. Gadgeel SM, Boinpally RR, Heilbrun LK, et al. A phase I clinical trial of spicamycin derivative KRN5500 (NSC 650426) using a phase I accelerated titration "2B" design. *Invest New Drugs*. 2003;21:63-74.
28. Gadgeel SM, Wozniak A, Boinpally RR, et al. Phase I clinical trial of BMS-247550, a derivative of epothilone B, using accelerated titration 2B design. *Clin Cancer Res*. 2005;11:6233-6239.
29. LoRusso PM, Foster BJ, Wozniak A, et al. Phase I pharmacokinetic study of the novel antitumor agent SR233377. *Clin Cancer Res*. 2000;6:3088-3094.
30. LoRusso PM, Prakash S, Wozniak A, et al. Phase I clinical trial of 5-fluoropyrimidinone (5FP), an oral prodrug of 5-fluorouracil (5FU). *Invest New Drugs*. 2002;20:63-71.
31. Eisenhauer EA. Phase I and II trials of novel anti-cancer agents: endpoints, efficacy and existentialism. The Michel Clavel Lecture, held at the 10th NCI-EORTC Conference on New Drugs in Cancer Therapy, Amsterdam, 16-19 June 1998. *Ann Oncol*. 1998;9:1047-1052.
32. Hunsberger S, Rubinstein LV, Dancey J, Korn EL. Dose escalation trial designs based on a molecularly targeted endpoint. *Stat Med*. 2005;24:2171-2181.
33. Korn EL. Nontoxicity endpoints in phase I trial designs for targeted, noncytotoxic agents. *J Natl Cancer Inst*. 2004;96:977-978.
34. Zhang W, Sargent DJ, Mandrekar S. An adaptive dose-finding design incorporating both toxicity and efficacy. *Stat Med*. 2006;25:2365-2383.
35. Collins JM, Grieshaber CK, Chabner BA. Pharmacologically guided phase I clinical trials based upon preclinical drug development. *J Natl Cancer Inst*. 1990;82:1321-1326.
36. Collins JM. Innovations in phase 1 trial design: where do we go next? *Clin Cancer Res*. 2000;6:3801-3802.
37. Fuse E, Kobayashi T, Inaba M, Sugiyama Y. Prediction of the maximal tolerated dose (MTD) and therapeutic effect of anticancer drugs in humans: integration of pharmacokinetics with pharmacodynamics and toxicodynamics. *Cancer Treatment Rev*. 1995;21:133-157.
38. Dees EC, Whitfield LR, Grove WR, Rummel S, Grochow LB, Donehower RC. A phase I and pharmacologic evaluation of the DNA intercalator CI-958 in patients with advanced solid tumors. *Clin Cancer Res*. 2000;6:3885-3894.
39. O'Quigley J, Pepe M, Fisher L. Continual reassessment method: a practical design for phase 1 clinical trials in cancer. *Biometrics*. 1990;46:33-48.
40. Garrett-Mayer E. The continual reassessment method for dose-finding studies: a tutorial. *Clin Trials (Lond)*. 2006;3:57-71.
41. Ivanova A, Montazer-Haghighi A, Mohanty SG, Durham SD. Improved up-and-down designs for phase I trials. *Stat Med*. 2003;22:69-82.
42. Rosenberger WF, Haines LM. Competing designs for phase I clinical trials: a review. *Stat Med*. 2002;21:2757-2770.
43. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med*. 2005;353:1659-1672.
44. Kabbinavar F, Hurwitz HI, Fehrenbacher L, et al. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol*. 2003;21:60-65.
45. Dancey JE, Chen HX. Strategies for optimizing combinations of molecularly targeted anticancer agents. *Nat Rev*. 2006;5:649-659.
46. Davis DW, McConkey DJ, Abbruzzese JL, Herbst RS. Surrogate markers in antiangiogenesis clinical trials. *Br J Cancer*. 2003;89:8-14.
47. Dowlati A, Haaga J, Remick SC, et al. Sequential tumor biopsies in early phase clinical trials of anticancer agents for pharmacodynamic evaluation. *Clin Cancer Res*. 2001;7:2971-2976.

48. Rimm DL. What brown cannot do for you. *Nat Biotechnol*. 2006;24:914-916.
49. Davis DW, Takamori R, Raut CP, et al. Pharmacodynamic analysis of target inhibition and endothelial cell death in tumors treated with the vascular endothelial growth factor receptor antagonists SU5416 or SU6668. *Clin Cancer Res*. 2005;11(2 Pt 1):678-689.
50. Davis DW, Shen Y, Mullani NA, et al. Quantitative analysis of biomarkers defines an optimal biological dose for recombinant human endostatin in primary human tumors. *Clin Cancer Res*. 2004;10(1 Pt 1):33-42.
51. Herbst RS, Mullani NA, Davis DW, et al. Development of biologic markers of response and assessment of antiangiogenic activity in a clinical trial of human recombinant endostatin. *J Clin Oncol*. 2002;20:3804-3814.
52. Shaheen RM, Davis DW, Liu W, et al. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res*. 1999;59:5412-5416.
53. Berger AJ, Davis DW, Tellez C, et al. Automated quantitative analysis of activator protein-2alpha subcellular expression in melanoma tissue microarrays correlates with survival prediction. *Cancer Res*. 2005;65:11185-11192.
54. Cregger M, Berger AJ, Rimm DL. Immunohistochemistry and quantitative analysis of protein expression. *Arch Pathol Lab Med*. 2006;130:1026-1030.
55. Xiong HQ, Herbst R, Faria SC, et al. A phase I surrogate endpoint study of SU6668 in patients with solid tumors. *Invest New Drugs*. 2004;22:459-466.
56. Davis DW, Buchholz TA, Hess KR, Sahin AA, Valero V, McConkey DJ. Automated quantification of apoptosis after neoadjuvant chemotherapy for breast cancer: early assessment predicts clinical response. *Clin Cancer Res*. 2003;9:955-960.
57. Davis DW, McConkey DJ, Heymach JV, et al. Pharmacodynamic analysis of target receptor tyrosine kinase activity and apoptosis in GIST tumors responding to therapy with SU11248. *Proc Am Soc Clin Oncol*. 2005:23 Abstr 3006.
58. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol*. 2006;33:369-385.
59. Hubbard SR. Structural analysis of receptor tyrosine kinases. *Prog Biophys Mol Biol*. 1999;71:343-358.
60. Bernatchez PN, Soker S, Sirois MG. Vascular endothelial growth factor effect on endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1-dependent. *J Biol Chem*. 1999;274:31047-31054.
61. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol*. 2003;21:2237-2246.
62. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA*. 2003;290:2149-2158.
63. Gasparini G, Sarmiento R, Amici S, et al. Gefitinib (ZD1839) combined with weekly epirubicin in patients with metastatic breast cancer: a phase I study with biological correlate. *Ann Oncol*. 2005;16:1867-1873.
64. Albanell J, Rojo F, Averbuch S, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J Clin Oncol*. 2002;20:110-124.
65. Von Hoff DD, LoRusso PM, Rudin CM, et al. Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *N Engl J Med*. 2009;361:1164-1172.
66. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
67. Kwak EL, Camidge DR, Clark J, et al. Clinical activity observed in a phase I dose escalation trial of an oral c-met and ALK inhibitor, PF-02341066. *ASCO Meeting Abstracts*. 2009;27(15S):3509.
68. Bollag G, Hirth P, Tsai J, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature*. 2010;467:596-599.
69. Lockhart AC, Rothenberg ML, Dupont J, et al. Phase I study of intravenous vascular endothelial growth factor trap, aflibercept, in patients with advanced solid tumors. *J Clin Oncol*. 2010;28:207-214.
70. Tew WP, Gordon M, Murren J, et al. Phase 1 study of aflibercept administered subcutaneously to patients with advanced solid tumors. *Clin Cancer Res*. 2010;16:358-366.
71. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011;364:2507-2516.
72. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010;363:1693-1703.
73. O'Hara SM, Moreno JG, Zweitzig DR, Gross S, Gomella LG, Terstappen LW. Multigene reverse transcription-PCR profiling of circulating tumor cells in hormone-refractory prostate cancer. *Clin Chem*. 2004;50:826-835.
74. Gordon. Phase I trial of recombinant humanized monoclonal anti-vascular endothelial growth factor (anti-VEGF MAB) in patients (PTS) with metastatic cancer (Meeting abstract) 1998.
75. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*. 2001;69:89-95.
76. Frank R, Hargreaves R. Clinical biomarkers in drug discovery and development. *Nat Rev*. 2003;2:566-580.
77. Collins JM. Functional imaging in phase I studies: decorations or decision making? *J Clin Oncol*. 2003;21:2807-2809.
78. Bergstrom M, Grahnen A, Langstrom B. Positron emission tomography microdosing: a new concept with application in tracer and early clinical drug development. *Eur J Clin Pharmacol*. 2003;59:357-366.
79. Galbraith SM. MR in oncology drug development. *NMR Biomed*. 2006;19:681-689.
80. Kelloff GJ, Hoffman JM, Johnson B, et al. Progress and promise of FDG-PET imaging for cancer patient management and oncologic drug development. *Clin Cancer Res*. 2005;11:2785-2808.
81. Mochizuki T, Tsukamoto E, Kuge Y, et al. FDG uptake and glucose transporter subtype expressions in experimental tumor and inflammation models. *J Nucl Med*. 2001;42:1551-1555.
82. de Geus-Oei L-F, van Krieken JH, Aliredjo RP, et al. Biological correlates of FDG uptake in non-small cell lung cancer. *Lung Cancer*. 2007;55:79-87.
83. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med*. 2002;347:472-480.
84. Prior JO, Montemurro M, Orcurto MV, et al. Early prediction of response to sunitinib after imatinib failure by ^{18}F-fluorodeoxyglucose positron emission tomography in patients with gastrointestinal stromal tumor. *J Clin Oncol*. 2009;27:439-445.
85. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors National Cancer Institute of the United States, National Cancer Institute of Canada European Organization for Research and Treatment of Cancer. *J Natl Cancer Inst*. 2000;92:205-216.
86. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45:228-247.
87. Husband JE, Schwartz LH, Spencer J, et al. Evaluation of the response to treatment of solid tumours—a consensus statement of the International Cancer Imaging Society. *Br J Cancer*. 2004;90:2256-2260.
88. Twombly R. Criticism of tumor response criteria raises trial design questions. *J Natl Cancer Inst*. 2006;98:232-234.
89. Schwartz LH, Mazumdar M, Wang L, et al. Response assessment classification in patients with advanced renal cell carcinoma treated on clinical trials. *Cancer*. 2003;98:1611-1619.
90. Moertel CG, Hanley JA. The effect of measuring error on the results of therapeutic trials in advanced cancer. *Cancer*. 1976;38:388-394.
91. Michaelis LC, Ratain MJ. Measuring response in a post-RECIST world: from black and white to shades of grey. *Nat Rev Cancer*. 2006;6:409-414.
92. Lavin PT. An alternative model for the evaluation of antitumor activity. *Cancer Clin Trials*. 1981;4:451-457.
93. Verweij J, Therasse P, Eisenhauer E. Cancer clinical trial outcomes: any progress in tumour-size assessment? *Eur J Cancer*. 2009;45:225-227.

Peter H. O'Donnell and Mark J. Ratain

49 Pharmacogenomics

Introduction

The term *pharmacogenetics* is believed to have first been used in 1959 by Vogel who defined it as "the study of genetically determined variations revealed by the effect of drugs."[1] Motulsky described the field similarly, but perhaps from the viewpoint of the clinical observer, calling the interaction "drug responses and their modification by hereditary influences."[2] With the advent of the ability to sequence the entire human genome[3,4]—wherein technology allows one the ability to rapidly and accurately determine billions of pieces of genetic information on a given individual—the term *pharmacogenetics* has slowly been replaced with *pharmacogenomics* to denote the consideration of the entire genome. Notwithstanding the term employed, the field is as relevant as ever. It offers the promise of achieving a paradigm of personalized drug therapy based on an individual's unique genetic makeup.

In reality, personalized care has been practiced for centuries, if one defines personalization as the consideration of all clinical, biologic, and environmental factors that make each patient unique. What changed with the sequencing of the first human genome—and through the simultaneous technological advances of profiling individuals (and, in cancer, tumors) on many levels (DNA, RNA, proteins, epigenetics)—was the expectation that "personalized medicine" would now also mean inclusion of molecular information. Some have termed this type of care *precision medicine*.[5] Pharmacogenomics, using that historical view, is therefore one of the oldest and most foundational pieces of precision medicine and precision therapeutics. Yet from the standpoint of how much there is still to know and implement, even after 50 years, pharmacogenomics is in its relative infancy.

Pharmacogenomics in Oncology

In cancer therapeutics, pharmacogenomic considerations are particularly relevant, for three reasons. First, many oncology drugs can have severe, undesirable toxicities. If genetic determinants or predictors of such toxicity could be identified, it might be possible to dramatically decrease undesirable harm when prescribing chemotherapy. Second, the stakes in cancer therapeutics are high. Lack of efficacy of a given treatment means loss of time, which in cancer care often is of seminal importance. Finally, pharmacogenomic considerations in oncology are complicated by the fact that there are two genomes in play: the genome of the patient (the germline or inherited DNA) and the genome of the tumor (the somatic or cancer DNA). Pharmacogenomic study has come to encompass the consideration of both, and thus it includes single-nucleotide polymorphisms (SNPs), gene copy number alterations (copy number variants, CNVs), and acquired changes (tumor mutations) as they relate to drug response or toxicity.[6,7] Somatic changes are of clear importance, including several that have led to the development of successful therapeutic interventions against a mutational event. However, for the scope of this chapter, we specifically restrict our considerations to germline genetic changes, because somatic changes reflect disease subcategorization at the molecular level, which is distinct from interindividual variability in drug response. In addition, in contrast to disease genetics, pharmacogenomics focuses specifically on predictive genetic markers of outcome from drug interventions, so we do not discuss DNA changes that potentially confer cancer susceptibility.

Even when considering only germline changes, pharmacogenomic questions have broad implications for oncology therapeutics in three very important ways:

1. In oncology, the concept of the therapeutic index is crucial. Many chemotherapy drugs were initially identified through screens for compounds that killed dividing cells.[8] Such drugs are still the mainstay of most anticancer regimens, but these cytotoxic drugs (and also even newer anticancer drugs that may work differently) are limited by possessing a narrow therapeutic index: the ratio between the dose that results in toxicity to the majority of individuals taking the drug, compared to the dose that results

in effect in the majority of individuals taking the drug. It is therefore rational to consider pharmacogenomic factors as potential determinants for drug dosing for chemotherapy drugs, in which relatively small changes in the dose can have dramatic undesirable effects (shifting the therapeutic index toward toxicity). Examples of this are provided later in the chapter.

2. In related fashion, it might be straightforward to understand how germline pharmacogenomic variants can be relevant for predicting the likelihood of toxicity from a drug, because germline variation in genes important for drug metabolism and excretion in the host might directly confer differences in enzyme function within these pathways. Indeed, many of the current, best studied examples of pharmacogenomic findings in oncology relate to toxicity predilection.[9] However, it has been interesting to also find that germline polymorphisms may affect *response* to treatment in malignancies. We explore ways that this occurs in this chapter. The important point is that germline pharmacogenomics has concrete promise for defining both *toxicity* and *response* in cancer therapeutics.
3. Germline pharmacogenomic discoveries have the potential to identify new oncology drug targets. Although this area remains one of ongoing research, germline mutations such as *BRCA1* may allow individuals with cancer to be more susceptible to certain types of DNA-damaging agents, such as poly(ADP-ribose)polymerase (PARP) inhibitors.[10,11] While drugs identified in this way remain under development, the number of examples like this is likely to increase, especially as more is learned about inherited changes in DNA structure and variation.

In this chapter we illustrate explicit examples of the first two points. However, we encourage the reader to simultaneously consider how, from the examples provided, opportunities for new drug discovery/identification of new drug targets (point 3) can also arise through the study of pharmacogenomics.

Genotyping and Phenotyping

Pharmacogenomic discovery and clinical implementation require the technical ability to accurately measure genotypes—the DNA sequence variation at specific genetic loci or regions. Most germline DNA variations fall into the class of SNPs, or single base-pair changes that occur with some relative prevalence in the human population (for example, a change from a guanine (G) to adenine (A) in the DNA strand [i.e., G/A polymorphism] in more than 1% of all individuals). Numerous technologies have been developed to perform genotyping and decipher such information at the DNA level.[12] Combining genetic data and associating it with a phenotype—an outward characteristic or trait—is where genomic information becomes valuable. Genotype-to-phenotype associations lie at the heart of pharmacogenomics, wherein all phenotypes of interest relate to drug effects (toxicities, response, drug levels in the blood, or drug effects on other biomarkers of drug action in the body). Clinical examples might include extreme drug phenotypes such as death, intermediate phenotypes such as development of severe hypertension, or more subtle phenotypes such as drug-induced hyperglycemia. The importance of accurate phenotyping also cannot be understated. Pharmacogenomic associations may not be detectable if phenotypes are not carefully defined and measured (ideally prospectively), or if important covariates such as cumulative drug dose are not considered. Similarly, relevant pharmacogenomic effects could be obscured by inappropriately combining phenotypes that are actually distinct.[13]

Pharmacogenomic Discovery Approaches

Single-Gene Approaches

Initial pharmacogenomic discovery methods typically consisted of "single-gene" approaches. These approaches presuppose that a given single gene (with purported a priori importance) is relevant to that drug's metabolism or action. Typically, these candidate genes code for enzymes involved in the mechanistic pathway of a drug's action or its metabolism. Variants within the candidate gene are then genotyped and analyzed in relation to a given drug phenotype.

The paradigmatic story of irinotecan pharmacogenomics is a classic single-gene example. Irinotecan is an intravenous chemotherapy drug first studied clinically in the late 1980s.[14,15] It is now used widely in the treatment of colorectal cancer. Importantly, irinotecan is a prodrug, requiring activation (through the action of carboxylesterase-2) to SN-38, an inhibitor of topoisomerase I, and then inactivation to SN-38 glucuronide (SN-38G) for elimination.[16,17] The stage for the pharmacogenomic story began with a phase I study conducted at The University of Chicago, which examined irinotecan on a weekly schedule and which suggested that the extent of glucuronidation to SN-38G was inversely correlated with irinotecan's dose-limiting toxicity.[18] Based on the fact that pharmacogenetic variation had been suggested to be related to toxicity for a few other cancer drugs at the time,[19-21] it was hypothesized that genetic factors resulting in reduced glucuronidation may be a cause of predisposition to irinotecan toxicity.[18] Studies in liver tissue from patients with a genetic deficiency of bilirubin

glucuronidation (Crigler-Najjar syndrome) were conducted, and it was found that these liver tissues were unable to form SN-38G.[22] However, transfection with cDNAs encoding the enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1) resulted in SN-38G formation.[22] This result was intriguing, because a genetic polymorphism in *UGT1A1* had recently been identified that was associated with variation in glucuronidation.[23,24] The polymorphism is now known to be an insertion/deletion in the *UGT1A1* promoter (the "TATA" box) that reduces transcription of the gene, such that the resulting enzyme activity is inversely correlated to the number of TA repeats.[25] Six repeats is considered the wild-type (normal function) genotype, and individuals typically have either 6 or 7 repeats, most commonly (5 and 8 are rare).[26] Using a special pharmacogenomic nomenclature, the 6-repeat genotype is termed **1*, whereas having 7 repeats is called **28*.

Given that there was a candidate polymorphism, an in vitro pharmacogenetic study was next conducted. Liver tissues were again used to test the hypothesis that genetic variability in the *UGT1A1* promoter was associated with SN-38G formation (which it was),[27] and then, importantly, with irinotecan toxicity in patients being treated with the drug.[28] The latter was further demonstrated (specifically an association with neutropenia from irinotecan) in a larger study of irinotecan-treated patients (350 mg/m^2 every 3 weeks).[29] Severe neutropenia was significantly more common in patients with two copies of the **28* variant (**28/*28*; 50% of such patients had severe neutropenia) compared to only 12.5% in those with a **1/*28* genotype, and no patients with severe neutropenia among those having **1/*1* genotypes. The relative risk of severe neutropenia was 9.3 (95% confidence interval 2.4-36.4) for the **28/*28* patients.[29] The authors[29] and, later, others[30] also confirmed the metabolic role of UGT1A1 with supportive pharmacokinetic information.

Based on the data, the FDA drug label for irinotecan was revised in 2005 to include the pharmacogenomic information about *UGT1A1*. It recommends that patients with the **28/*28* genotype should be treated with a reduced starting dose of irinotecan.[31]

A large study including 250 metastatic colorectal cancer patients went on to redemonstrate the pharmacogenomic importance of *UGT1A1*, finding an odds ratio of risk of approximately 9 for those with the highest risk genotype (**28/*28*) for developing immediate severe neutropenia after irinotecan, although the pharmacogenomic relationship did not persist for subsequent treatment cycles of irinotecan.[32] A meta-analysis of the data was performed and published in 2007.[33] The meta-analysis included nine studies and a total of 821 patients. It confirmed the importance of **28* homozygosity. However, it interestingly suggested that the pharmacogenomic effect was most important for patients receiving higher doses of irinotecan (150 mg/m^2 or higher). At lower doses (doses that are still in the commonly used range), there was no statistically significant increased risk for patients with the **28/*28* genotype.

Given these nuances and concerns about the predictive power of irinotecan pharmacogenomics during initial dosing, some have argued that *UGT1A1* genotyping cannot be justified as a mandatory test in every patient for the purpose of initial dosage adjustment.[34] Indeed, *UGT1A1 *28* does not meet the typical standards for clinical specificity and sensitivity required of most diagnostic tests (one analysis suggested that its median positive predictive value was 50% [range 15% to 57%] and median negative predictive value was 85% [range 79% to 96%]).[26] Moreover, the **28* genotype alone is not sufficient to describe risk in individuals of some ethnic backgrounds, since the **6* allele also results in decreased UGT1A1 activity.[26,35] Nonetheless, the test clearly has potential clinical value in ethnic groups with a high prevalence of the **28* genotype and when high doses of irinotecan are planned, but these latter considerations emphasize the need for ongoing study of ways to improve the understanding of toxicity risk with irinotecan administration. Including other risk alleles within *UGT1A* in a haplotype-based analysis may increase the predictive value, because several other variants in these genes have now also been shown to alter enzymatic activity and affect irinotecan-related outcomes.[36]

Pathway Approaches

Pathway approaches use the rationale that a single gene may be of limited lone importance from a pharmacogenomic standpoint, because few drugs are metabolized by or have mechanisms involving only one gene product. In other words, most drugs are likely to be under multigenic control.[37] Therefore, pathway approaches hypothesize that a set of genes defining a specific metabolic or mechanistic pathway might sensibly be interrogated as a composite set, for pharmacogenomic relevance. Typically, investigators will select various polymorphisms of interest that have potential biologic relevance in the genes of interest (e.g., exon SNPs that change the coding sequence, or SNPs in promoter regions)—or, less specifically, they will select SNPs that "tag" regions across the genes of interest. Despite the reasonable rationale, to our knowledge this approach to pharmacogenomics has yet to yield any salient, clinically important results. Such studies often suffer from the problem that multiple SNPs are being tested, meaning that there is limited power for demonstrating statistical significance of any one SNP in small sample sizes (see further discussion of this later).

Genome-Wide Approaches

Single-gene approaches like those described earlier have the advantage that the analyses are conducted on very focused areas of the genome, areas having potentially the highest relevance. Typically, some evidence already exists that the gene is important on a functional level, usually through studies showing that the gene product is under differential expression or that the gene product is key to determining the pharmacokinetics of a drug. When only a single gene or even a few genes (as in pathway approaches) are being considered, only a limited amount of genomic information must be tested and assimilated using bioinformatic techniques.

In contrast, genome-wide association studies (GWAS) take an unbiased approach to pharmacogenomic discovery. These are open to the possibility that any gene is important (unbiased) and they can identify new genetic targets (i.e., they are hypothesis generating). The limitations of GWAS include a need for vast computational ability, the possibility of false discovery (explained further later), and, typically, a requirement for genetic information on a large number of individuals to identify compelling associations.

One well-executed example of the power of this approach is illustrated by the work of Yang and colleagues.[38] The authors set out to identify germline genetic factors that might predict therapy response in pediatric acute lymphoblastic leukemia (ALL). They and others had observed that significant interindividual heterogeneity existed for treatment response in ALL, and that most prior work had focused on tumor-related factors which might explain such variation. The authors hypothesized that unidentified germline (host) factors might also be important. Indeed, one pharmacogenomic factor had already been well described for a drug commonly used to treat ALL and other leukemias—6-mercaptopurine—although the pharmacogenomic relationship was that of the role of TPMT (thiopurine methyltransferase) activity in predicting severe toxicity (bone marrow suppression) from that drug,[39,40] not treatment response. The story of TPMT/6-mercaptopurine (and, relatedly, TPMT/thioguanine) toxicity pharmacogenetics is one of the earliest and best-characterized examples of the potential utility of pharmacogenomics. The FDA labels for 6-mercaptopurine and thioguanine have been revised to include TPMT pharmacogenetic information,[41] and some hospitals have implemented routine ordering of TPMT testing before administration of these agents.[42] This pharmacogenomic relationship had been identified through a candidate gene approach.

Yang and associates, however, wanted to determine whether there were germline factors predictive of treatment response in ALL.[28] Rather than restricting their analysis to any one candidate gene, they performed a GWAS of more than 470,000 germline SNPs to identify genotypes that associated with increased risk of minimal residual disease (MRD) in two independent cohorts (318 children and 169 children) with newly diagnosed ALL. They identified 102 SNPs associated with MRD in both cohorts.[38] Twenty-one of these SNPs were also associated with antileukemic drug disposition, strengthening a plausible link between MRD eradication and greater drug exposure. Many SNPs were in regions never previously studied as potential pharmacogenomic loci. Interestingly, five of the top associated SNPs were located in the interleukin 15 gene (*IL-15*) gene.[38] IL-15 was previously shown to protect lymphoid tumors from glucocorticoid-induced apoptosis in vitro,[43] and IL-15 expression in ALL blasts had been linked to central nervous system (CNS) involvement at diagnosis and an increased risk of CNS relapse.[44] Therefore, the link between germline SNPs in *IL-15* and worse outcomes, for several possible reasons, seemed strong. Because the authors did not (for obvious reasons) have an untreated control group in their study, it was impossible to say whether these *IL-15* germline SNPs were truly pharmacogenomic SNPs (predictive of residual disease through their action on antileukemic drug action) or prognostic SNPs that conferred a worse outcome, regardless of treatment. This points to how GWAS approaches can, simultaneously, yield powerful results and be hypothesis-generating, encouraging further follow-up of the role of IL-15 in this disease and its treatment. This point notwithstanding, at least some of the top 102 SNPs in this study were linked to antileukemic drug disposition and can therefore be characterized as pharmacogenomic, showing the power of this approach as a pharmacogenomic discovery tool.

Whole-Genome Sequencing (WGS)

Very recently, WGS methods have begun to become more commonplace.[45] This approach has the advantage of theoretically identifying every piece of genetic information (and also variation) across the entire genome, including every base pair, deletion/insertion, and copy number change. GWAS approaches, in contrast, classically cover only a limited number of such variants across the entire genome. Concrete examples of applications of WGS to pharmacogenomics are still currently awaited.

Bioinformatic Considerations

Last, it is important to describe the potential for false discovery in pharmacogenomic analyses. This is the inherent possibility that a variant will be found to be statistically

associated with a phenotype by chance alone.[46-48] When one is assimilating millions or even billions of pieces of genetic information at once (as in GWAS and WGS methods), the sheer number of association tests being performed is so large that one must adjust the threshold of statistical significance that defines a positive result.[46] As an additional measure of sorting true-positive findings from false positives, it is typically accepted that high-quality pharmacogenomic results are those for which the association can be replicated in an independent population.[49] Here again, assurance that the phenotypes match and were collected in similar fashion is an important methodological step.

Clinical Relevance of Pharmacogenomic Findings During Implementation

A number of oncology drugs have been studied to uncover pharmacogenomic effects,[9,34] and other well-validated oncology drug-gene interactions exist (TPMT/6-mercaptopurine; 5-fluorouracil/DPYD). An even greater number of promising associations have been described for many other drugs that await further characterization and validation.

What remains to be further elucidated for many of these associations is the degree to which individual associations have an effect on a clinical outcome when assimilated within the larger context of all variables that affect a therapeutic decision. By this we mean: when a clinician makes a prescribing choice, he or she already is used to—and is accomplished at—incorporating many factors to make the best drug choice. These include all of the elements of "traditional" personalized care: clinical factors about comorbidities, organ function, allergies, and drug-drug interactions; biologic factors such as disease subtype, age, and gender; and environmental factors such as habits, social environment, and compliance. The question is whether a specific pharmacogenomic association for a drug's toxicity or response likelihood—which is probabilistic by its very nature and is usually identified in a large cohort showing an overall effect—will matter for an individual patient when the clinician has to assimilate the pharmacogenomic information along with all other treatment factors, including potentially other important genetic factors. In many respects, it is a question that resolves around the effect size of the pharmacogenomic effect. The *UGT1A1* example—where the dose of the drug being administered (irinotecan) seems to matter as to whether the pharmacogenomic effect is observable[33]—may be one example of this dynamic and delicate interdependence between genetic effects and other clinical effects (such as dose) interacting (or one predominating) to determine the clinical phenotype.

A nononcology example here is perhaps illustrative. Using a genome-wide approach in a group of 1953 patients with major depressive disorder, researchers were interested in finding pharmacogenomic markers predictive of treatment response for the antidepressant drug citalopram.[50] Their results identified an SNP having an association with response with a P of approximately 1×10^{-6}. This SNP was then positively replicated by the same authors in a smaller, independent cohort of patients. Patients who were homozygous for the allele of interest had an 18% reduction in absolute risk of having no response to treatment.[50] Moreover, when this SNP was considered in a second analysis along with another SNP that had been independently associated with response likelihood, the combined reduction in risk (as one might predict) was even greater: 23%.[51] Despite these exciting findings, the authors examined their own data in another way to estimate the overall independent clinical predictive effect of using the genetic markers themselves. The authors found that it was, at best, modest, with a c-statistic of 0.58 using a receiver operator characteristic analysis, which expresses the probability of correctly identifying a responder from a random pair of patients.[51] This demonstrates that the effect of other clinical and environmental variables—besides pharmacogenomic variables—remains powerful. Others have argued this as well.[52] We believe that multivariate analyses which include genetic variables among other accepted clinical variables are what will be truly needed to demonstrate the clinical effectiveness of pharmacogenomic discoveries. In oncology, where another layer of potential deterministic relevance is involved (i.e., the tumor DNA), the link between a germline pharmacogenomic variant and a clinical outcome of interest is likely to be even more complex. Deciphering the stringency of such genotype-phenotype relationships in clinical use is one of the most pertinent questions currently facing pharmacogenomics.

Conclusions

Studies have led to the identification of genetic factors governing drug response and toxicity for hundreds of drugs,[53] including some chemotherapy drugs and agents used in the long-term therapy of cancer patients. Corresponding pharmacogenomic tests are available for clinical use for certain drugs, including chemotherapies, for which significant evidence has been accumulated, and FDA drug labels are increasingly incorporating consideration of pharmacogenomic factors in the prescribing information. Knowledge about pharmacogenomics can manifest as patient-specific dose modifications, optimization of

treatment choice when several equivalent therapies exist, or avoidance of a therapy when toxicity risks outweigh potential benefits. Even with this progress, important steps remain in navigating the translation of pharmacogenomic knowledge into widespread clinical use. However, precision medicine is unlikely to turn backward, and pharmacogenomics promises to be part of that evolution. On a population scale in oncology, it has the potential to reduce the significant burden of illness that is conferred when chemotherapies cause toxicity, or when chemotherapies do not work. In that regard, there are few more important questions facing oncology today.

Key References

5. Mirnezami R, Nicholson J, Darzi A. Preparing for precision medicine. *N Engl J Med*. 2012;366:489-491.
6. Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. *N Engl J Med*. 2011;364:1144-1153.
7. Watson RG, McLeod HL. Pharmacogenomic contribution to drug response. *Cancer J*. 2011;17:80-88.
8. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*. 2012;12:237-251.
9. O'Donnell PH, Ratain MJ. Germline pharmacogenomics in oncology: decoding the patient for targeting therapy. *Mol Oncol*. 2012;6:251-259.
10. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.
11. Postel-Vinay S, Vanhecke E, Olaussen KA, et al. The potential of exploiting DNA-repair defects for optimizing lung cancer treatment. *Nat Rev Clin Oncol*. 2012;9:144-155.
12. Tran B, Dancey JE, Kamel-Reid S, et al. Cancer genomics: technology, discovery, and translation. *J Clin Oncol*. 2012;30:647-660.
13. Johnson JA, Roden DM, Lesko LJ, et al. Clopidogrel: a case for indication-specific pharmacogenetics. *Clin Pharmacol Ther*. 2012;91:774-776.
29. Innocenti F, Undevia SD, Iyer L, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J Clin Oncol*. 2004;22:1382-1388.
30. Minami H, Sai K, Saeki M, et al. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet Genomics*. 2007;17:497-504.
31. Food and Drug Administration. *Revised Label for Camptosar*. In Edition 2006.
32. Toffoli G, Cecchin E, Corona G, et al. The role of UGT1A1*28 polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. *J Clin Oncol*. 2006;24:3061-3068.
33. Hoskins JM, Goldberg RM, Qu P, et al. UGT1A1*28 genotype and irinotecan-induced neutropenia: dose matters. *J Natl Cancer Inst*. 2007;99:1290-1295.
34. Lee SY, McLeod HL. Pharmacogenetic tests in cancer chemotherapy: what physicians should know for clinical application. *J Pathol*. 2011;223:15-27.
35. Han JY, Lim HS, Shin ES, et al. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. *J Clin Oncol*. 2006;24:2237-2244.
36. Cecchin E, Innocenti F, D'Andrea M, et al. Predictive role of the UGT1A1, UGT1A7, and UGT1A9 genetic variants and their haplotypes on the outcome of metastatic colorectal cancer patients treated with fluorouracil, leucovorin, and irinotecan. *J Clin Oncol*. 2009;27:2457-2465.
37. Kotti S, Bickeboller H, Clerget-Darpoux F. Strategy for detecting susceptibility genes with weak or no marginal effect. *Hum Hered*. 2007;63:85-92.
38. Yang JJ, Cheng C, Yang W, et al. Genome-wide interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia. *JAMA*. 2009;301:393-403.
39. Relling MV, Gardner EE, Sandborn WJ, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin Pharmacol Ther*. 2011;89:387-391.
41. Food and Drug Administration. *Table of Pharmacogenomic Biomarkers in Drug Labels*. In Edition 2012. http://www.fda.gov/%20drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm.
42. Marshall E. Preventing toxicity with a gene test. *Science*. 2003;302:588-590.
44. Cario G, Izraeli S, Teichert A, et al. High interleukin-15 expression characterizes childhood acute lymphoblastic leukemia with involvement of the CNS. *J Clin Oncol*. 2007;25:4813-4820.
45. Gonzaga-Jauregui C, Lupski JR, Gibbs RA. Human genome sequencing in health and disease. *Annu Rev Med*. 2012;63:35-61.
46. Rice TK, Schork NJ, Rao DC. Methods for handling multiple testing. *Adv Genet*. 2008;60:293-308.
47. van den Oord EJ. Controlling false discoveries in genetic studies. *Am J Med Genet B Neuropsychiatr Genet*. 2008;147B:637-644.
48. Maitland ML, Ratain MJ, Cox NJ. Interpreting P values in pharmacogenetic studies: a call for process and perspective. *J Clin Oncol*. 2007;25:4513-4515.
49. Chanock SJ, Manolio T, Boehnke M, et al. Replicating genotype-phenotype associations. *Nature*. 2007;447:655-660.
50. McMahon FJ, Buervenich S, Charney D, et al. Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am J Hum Genet*. 2006;78:804-814.
51. Paddock S, Laje G, Charney D, et al. Association of GRIK4 with outcome of antidepressant treatment in the STAR*D cohort. *Am J Psychiatry*. 2007;164:1181-1188.
52. Kitsios GD, Kent DM. Personalised medicine: not just in our genes. *BMJ*. 2012;344:e2161.

References

1. Vogel F. Moderne Probleme der Humangenetik. *Ergeb Inn Med Kinderheilkd*. 1959;12:52-125.
2. Motulsky AG. Pharmacogenetics. *Prog Med Genet*. 1964;23:49-74.
3. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860-921.
4. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science*. 2001;291:1304-1351.
5. Mirnezami R, Nicholson J, Darzi A. Preparing for precision medicine. *N Engl J Med*. 2012;366:489-491.
6. Wang L, McLeod HL, Weinshilboum RM. Genomics and drug response. *N Engl J Med*. 2011;364:1144-1153.
7. Watson RG, McLeod HL. Pharmacogenomic contribution to drug response. *Cancer J*. 2011;17:80-88.
8. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer*. 2012;12:237-251.
9. O'Donnell PH, Ratain MJ. Germline pharmacogenomics in oncology: decoding the patient for targeting therapy. *Mol Oncol*. 2012;6:251-259.
10. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.
11. Postel-Vinay S, Vanhecke E, Olaussen KA, et al. The potential of exploiting DNA-repair defects for optimizing lung cancer treatment. *Nat Rev Clin Oncol*. 2012;9:144-155.
12. Tran B, Dancey JE, Kamel-Reid S, et al. Cancer genomics: technology, discovery, and translation. *J Clin Oncol*. 2012;30:647-660.
13. Johnson JA, Roden DM, Lesko LJ, et al. Clopidogrel: a case for indication-specific pharmacogenetics. *Clin Pharmacol Ther*. 2012;91:774-776.
14. Slichenmyer WJ, Von Hoff DD. New natural products in cancer chemotherapy. *J Clin Pharmacol*. 1990;30:770-788.
15. Ohno R, Ota K, Masaoka T, et al. A clinical study of a camptothecin derivative, CPT-11, on hematological malignancies. *Proc Am Soc Clin Oncol*. 1989;8:261.
16. Tsuji T, Kaneda N, Kado K, et al. CPT-11 converting enzyme from rat serum: purification and some properties. *J Pharmacobiodyn*. 1991;14:341-349.
17. Atsumi R, Suzuki W, Hakusui H. Identification of the metabolites of irinotecan, a new derivative of camptothecin, in rat bile and its biliary excretion. *Xenobiotica*. 1991;21:1159-1169.
18. Gupta E, Lestingi TM, Mick R, et al. Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea. *Cancer Res*. 1994;54:3723-3725.
19. Lennard L, Van Loon JA, Weinshilboum RM. Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism. *Clin Pharmacol Ther*. 1989;46:149-154.
20. Harris BE, Carpenter JT, Diasio RB. Severe 5-fluorouracil toxicity secondary to dihydropyrimidine dehydrogenase deficiency. A potentially more common pharmacogenetic syndrome. *Cancer*. 1991;68:499-501.
21. Ratain MJ, Mick R, Berezin F, et al. Paradoxical relationship between acetylator phenotype and amonafide toxicity. *Clin Pharmacol Ther*. 1991;50:573-579.
22. Iyer L, King CD, Whitington PF, et al. Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes. *J Clin Invest*. 1998;101:847-854.
23. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med*. 1995;333:1171-1175.
24. Monaghan G, Ryan M, Seddon R, et al. Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet*. 1996;347:578-581.
25. Beutler E, Gelbart T, Demina A. Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? *Proc Natl Acad Sci U S A*. 1998;95:8170-8174.
26. Innocenti F, Ratain MJ. Pharmacogenetics of irinotecan: clinical perspectives on the utility of genotyping. *Pharmacogenomics*. 2006;7:1211-1221.
27. Iyer L, Hall D, Das S, et al. Phenotype-genotype correlation of in vitro SN-38 (active metabolite of irinotecan) and bilirubin glucuronidation in human liver tissue with UGT1A1 promoter polymorphism. *Clin Pharmacol Ther*. 1999;65:576-582.
28. Iyer L, Das S, Janisch L, et al. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. *Pharmacogenomics J*. 2002;2:43-47.
29. Innocenti F, Undevia SD, Iyer L, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J Clin Oncol*. 2004;22:1382-1388.
30. Minami H, Sai K, Saeki M, et al. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet Genomics*. 2007;17:497-504.
31. Food and Drug Administration. *Revised Label for Camptosar*. In Edition 2006.
32. Toffoli G, Cecchin E, Corona G, et al. The role of UGT1A1*28 polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. *J Clin Oncol*. 2006;24:3061-3068.
33. Hoskins JM, Goldberg RM, Qu P, et al. UGT1A1*28 genotype and irinotecan-induced neutropenia: dose matters. *J Natl Cancer Inst*. 2007;99:1290-1295.
34. Lee SY, McLeod HL. Pharmacogenetic tests in cancer chemotherapy: what physicians should know for clinical application. *J Pathol*. 2011;223:15-27.
35. Han JY, Lim HS, Shin ES, et al. Comprehensive analysis of UGT1A polymorphisms predictive for pharmacokinetics and treatment outcome in patients with non-small-cell lung cancer treated with irinotecan and cisplatin. *J Clin Oncol*. 2006;24:2237-2244.
36. Cecchin E, Innocenti F, D'Andrea M, et al. Predictive role of the UGT1A1, UGT1A7, and UGT1A9 genetic variants and their haplotypes on the outcome of metastatic colorectal cancer patients treated with fluorouracil, leucovorin, and irinotecan. *J Clin Oncol*. 2009;27:2457-2465.
37. Kotti S, Bickeboller H, Clerget-Darpoux F. Strategy for detecting susceptibility genes with weak or no marginal effect. *Hum Hered*. 2007;63:85-92.
38. Yang JJ, Cheng C, Yang W, et al. Genome-wide interrogation of germline genetic variation associated with treatment response in childhood acute lymphoblastic leukemia. *JAMA*. 2009;301:393-403.
39. Relling MV, Gardner EE, Sandborn WJ, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. *Clin Pharmacol Ther*. 2011;89:387-391.
40. Evans WE. Thiopurine S-methyltransferase: a genetic polymorphism that affects a small number of drugs in a big way. *Pharmacogenetics*. 2002;12:421-423.
41. Food and Drug Administration. *Table of Pharmacogenomic Biomarkers in Drug Labels*. In Edition 2012. http://www.fda.gov/%20drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm.
42. Marshall E. Preventing toxicity with a gene test. *Science*. 2003;302:588-590.
43. Tinhofer I, Marschitz I, Henn T, et al. Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma. *Blood*. 2000;95:610-618.
44. Cario G, Izraeli S, Teichert A, et al. High interleukin-15 expression characterizes childhood acute lymphoblastic leukemia with involvement of the CNS. *J Clin Oncol*. 2007;25:4813-4820.
45. Gonzaga-Jauregui C, Lupski JR, Gibbs RA. Human genome sequencing in health and disease. *Annu Rev Med*. 2012;63:35-61.
46. Rice TK, Schork NJ, Rao DC. Methods for handling multiple testing. *Adv Genet*. 2008;60:293-308.
47. van den Oord EJ. Controlling false discoveries in genetic studies. *Am J Med Genet B Neuropsychiatr Genet*. 2008;147B:637-644.
48. Maitland ML, Ratain MJ, Cox NJ. Interpreting P values in pharmacogenetic studies: a call for process and perspective. *J Clin Oncol*. 2007;25:4513-4515.
49. Chanock SJ, Manolio T, Boehnke M, et al. Replicating genotype-phenotype associations. *Nature*. 2007;447:655-660.
50. McMahon FJ, Buervenich S, Charney D, et al. Variation in the gene encoding the serotonin 2A receptor is associated with outcome of antidepressant treatment. *Am J Hum Genet*. 2006;78:804-814.
51. Paddock S, Laje G, Charney D, et al. Association of GRIK4 with outcome of antidepressant treatment in the STAR*D cohort. *Am J Psychiatry*. 2007;164:1181-1188.
52. Kitsios GD, Kent DM. Personalised medicine: not just in our genes. *BMJ*. 2012;344:e2161.
53. Hewett M, Oliver DE, Rubin DL, et al. PharmGKB: the Pharmacogenetics Knowledge Base. *Nucleic Acids Res*. 2002;30:163-165.

Louis M. Weiner and Rishi Surana

50

Monoclonal Antibodies for the Treatment of Cancer

Introduction

Monoclonal antibodies have emerged as mainstays of cancer therapy and have had a significant impact on the morbidity and mortality of several cancers. This chapter will discuss the attributes that make antibodies powerful cancer therapeutics, how some antibodies are used clinically, and the development of new antibodies with clinical promise.

Antibody Structure and Function

Antibodies are heterodimeric proteins composed of two heavy chains and two light chains connected by disulfide bridges. The two light chains each contain one variable region (V_L) and one constant region (C_L), whereas each heavy chain contains one variable region (V_H) and up to three distinct constant regions (C_H1 to -3) (Figure 50-1). Differential utilization of C_H domains can be used to classify antibodies into five main groups, or *isotypes:* IgD, IgA, IgG, IgM, and IgE. For example, IgM uses heavy-chain constant domains from the $C_H\mu$ gene, whereas IgG uses the $C_H\gamma$ gene. IgG is the isotype most commonly used in cancer immunotherapy and is the focus of this chapter.

Structurally, antibodies can be divided into two functional modalities: the fragment of antigen binding (Fab) and the fragment of crystallization (Fc).

The Fab is responsible for antigen binding and is composed of the full-length light chain (V_L + C_L) and the V_H and C_H1 domains of the heavy chain. The V_L and V_H domains, collectively referred to as the *variable fragment* (Fv), contain six hypervariable regions called *complementary determining regions* (CDRs). The antigen binding pocket, also referred to as the *paratope*, is formed by opposition of three CDRs located on the V_L domain (CDR-L1, -L2, -L3) with three CDRs on the V_H domain (CDR-H1, -H2, -H3). The paratope of the antibody binds to a small region of approximately five to eight amino acids on the antigen called the *epitope*. A complex system of genetic recombinations and rearrangements is responsible for generating diversity among CDR sequences, resulting in a human antibody repertoire with between 10^{10} and 10^{11} distinct paratopes.[1]

The Fc portion of IgG antibodies is composed of C_H2 and C_H3 domains and is required to initiate effector immune responses such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). IgGs engage immune effector cells via Fc-γ receptors (FcγRs), which are expressed on a diverse range of hematopoietic cells. In humans, there are three activating FcγRs: FcγRI (CD64), FcγRIIA (CD32A), and FcγRIIIA (CD16). When engaged, these receptors transduce activating signals via immunoreceptor tyrosine-based activation motifs (ITAMs), resulting in initiation of ADCC or phagocytosis. Similarly, FcγRs can transduce inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The primary inhibitory receptor in humans is FcγRIIB (CD32) which is expressed on a wide range of effector cells, with the notable exception of natural killer (NK) cells. In addition, neutrophils express FcγRIIIB, which is a GPI-linked isoform that serves as a decoy receptor and a sink for immune complexes. IgGs can also bind to neonatal Fc receptors (FcRn), which mediates transplacental transfer of maternal antibodies to the fetus. FcRns are also expressed in the vascular endothelium, where they bind IgGs and return them to the circulation, thereby prolonging their serum half-life.[2]

Development of Monoclonal Antibodies

The introduction of hybridoma technology in 1975 by Kohler and Milstein allowed for the mass production of murine monoclonal antibodies.[3] This technology used

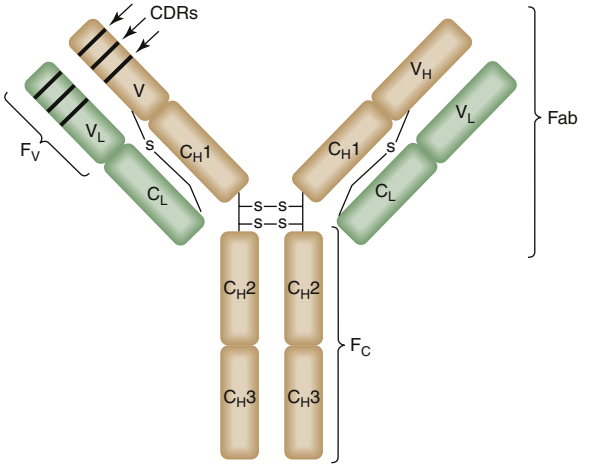


Figure 50-1 Structure of IgG.

murine B cells from immunized mice fused together with an immortalized murine plasma cell line. Through a series of selections and limiting dilutions, a clonal population could be isolated that produced antibodies specific for a single epitope. Eventually, antibodies to tumor antigens were developed and tested in clinical trials. The results of early trials demonstrated limited therapeutic benefit, primarily due to the production of human anti-murine antibodies (HAMAs), which resulted in rapid clearance of the drug and occasionally significant immune-mediated adverse events such as rash and renal failure.[4,5] In addition, the lower affinity of murine Fc to human FcRn may contribute to shorter serum half-life.[6] In the early 1980s, IgG genes were cloned and expressed in mammalian cells, paving the way for the development of chimeric antibodies, which contain murine variable regions and human constant regions.[7,8] Chimeric antibodies have reduced immunogenicity compared to their murine counterpart, but some patients still develop an immune response to the residual murine component (Figure 50-2). Humanized antibodies, which contain human heavy and light chains and murine CDRs, were developed in the late 1980s in an effort to further reduce immunogenicity.[9]

Currently, fully human antibodies are being produced using two common approaches: screening recombinant antibody libraries and engineering transgenic animals to express human immunoglobulin genes. A library of antibody fragments can be generated from human B cells, or by using cloning techniques, and can be used to construct phage or microbial display libraries. These libraries are subjected to multiple rounds of screening against the antigen of interest, and eventually high-affinity antibody fragments can be isolated.[10] These fragments can be used to generate full-length human antibodies.

More recently, transgenic mice expressing various human antibody gene sequences have been used to develop high-affinity, highly specific, fully human antibodies.[11] Panitumumab, an antibody targeting the epidermal growth factor receptor (EGFR), is an example of an antibody generated in transgenic animals and is currently approved for the treatment of refractory metastatic colon cancer.[12]

Mechanisms of Action of Anti-Cancer Antibodies

Signaling Perturbation

Solid tumors often upregulate expression of growth factor receptors or depend on these receptors for their proliferation or survival, with an enhanced capacity to invade and metastasize. Members of the epidermal growth factor (EGF) family of receptors, including EGFR1, HER2/neu (EGFR2), EGFR3, and EGFR4, are often overexpressed on solid tumors and thus serve as attractive targets for antibody therapy.[13] Most antibodies targeting EGFR family members work in part by inhibiting ligand binding and subsequent downstream oncogenic signaling (Figure 50-3). Notable exceptions are antibodies that target HER2/neu. Because this receptor has no known ligand, anti-HER2/neu antibodies such as trastuzumab and pertuzumab perturb signaling by preventing dimerization with other EGFR family members.[14]

ADCC

Several tumor-targeted monoclonal antibodies rely on activation of host immune responses, such as ADCC, in order to exert their full therapeutic benefit. Both IgG1 and IgG3 subclasses can bind with high affinity to FcγRs and initiate ADCC-mediated killing of tumor cell targets. Antibody-coated tumor cells bind to FcγRs on innate effector cells, primarily NK cells, macrophages, and neutrophils, leading to targeted release of cytoplasmic granules containing perforin and granzyme, resulting in tumor cell apoptosis (see Figure 50-3). Preclinical studies demonstrated that FcγRs are critically important for the antitumor activities of trastuzumab and the anti-CD20 antibody rituximab.[15] Furthermore, expression of inhibitory FcγRIIB limits the efficacy of trastuzumab and rituximab in vivo, suggesting that the balance between activating and inhibitory FcγRs is a critical determinant of the antitumor activity of these antibodies. Positive correlations between FcγRIIA-131H and FcγRIIIA-158V polymorphisms and clinical outcomes have been demonstrated in patients with follicular lymphoma treated with rituximab[16,17] and patients with refractory colorectal cancer treated with cetuximab.[18] Similarly, patients with metastatic breast cancer harboring FcγRIIA-131H and/or FcγRIIIA-158V polymorphisms show a greater response to trastuzumab-based treatment regimens compared to patients with

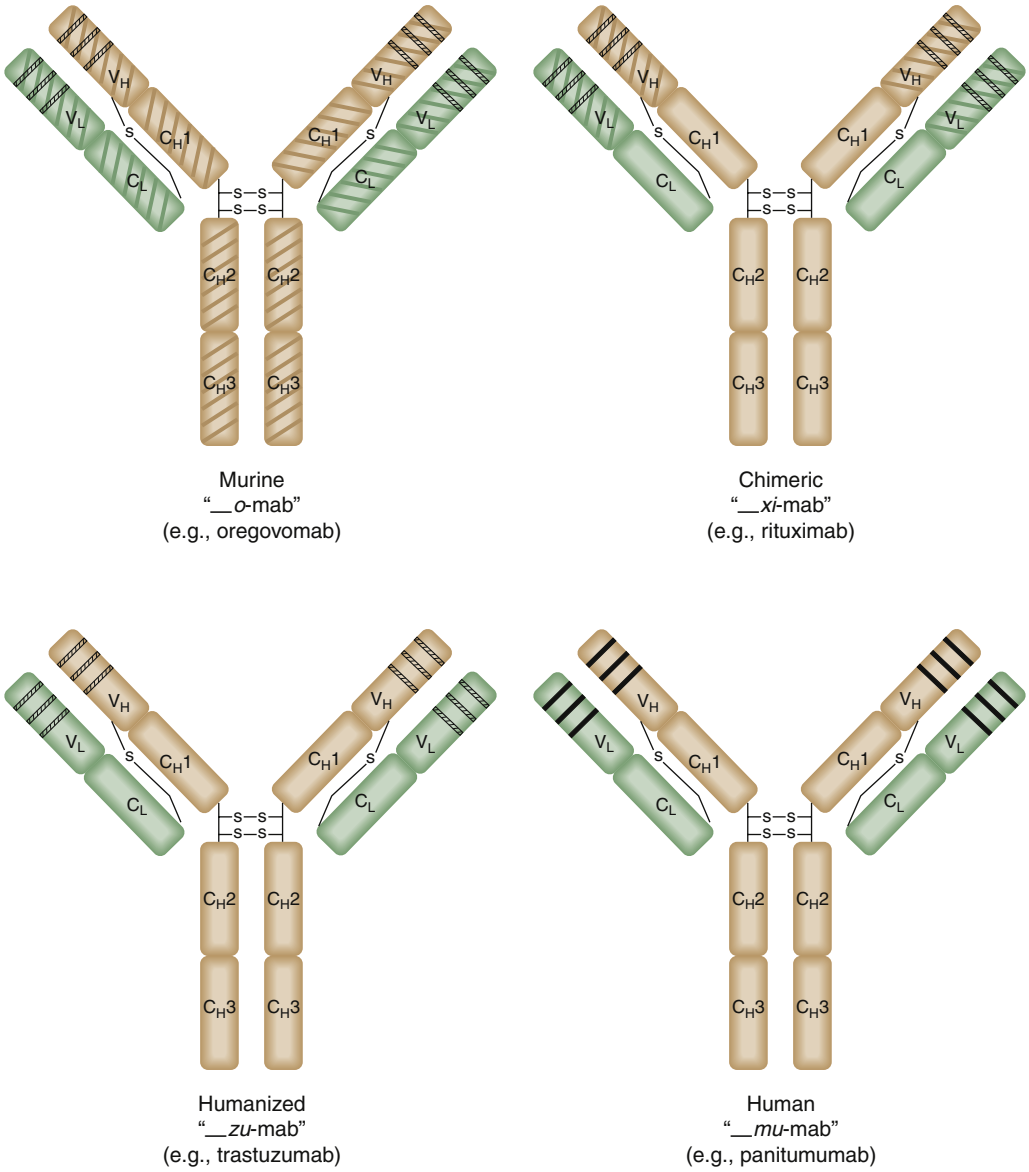


Figure 50-2 Nomenclature of monoclonal antibodies.

the 131R and/or 158F polymorphisms.[19] The FcγRIIIA-158V receptor has a higher affinity for human IgG1 and a greater capacity to mediate ADCC compared to the 158F receptor.[20,21]

CDC

In addition to binding FcγRs, IgG1 and IgG3 subclasses are potent activators of the classical complement cascade. The cascade is initiated with the binding of the C1q complex to the Fc domain of antibodies bound to tumor antigens. The C1q complex is composed of the hexameric C1q protein bound to the zymogens C1r and C1s. Binding of multiple C1q molecules activates its enzymatic activity, resulting in cleavage and activation of the C1s serine protease, which in turn activates downstream complement proteins culminating in the formation of the membrane attack complex (MAC). The MAC forms a pore in the lipid bilayer that compromises membrane integrity and causes target cell death. Certain complement protein fragments, such as C5a, induce a local inflammatory response by functioning as chemoattractants for neutrophils, monocytes, and lymphocytes. Rituximab is capable of mediating CDC against various B-cell malignancies in vitro in part because of its ability to translocate CD20 onto lipid raft microdomains, resulting in more efficient complement fixation.[22] The antitumor activity of rituximab is abolished in C1q knockout animals, supporting CDC as an important mechanism underlying the therapeutic efficacy of rituximab.[23] Clinically, however, the importance of CDC in the context of rituximab therapy is still undetermined. Patients with follicular lymphoma harboring the C1qA276

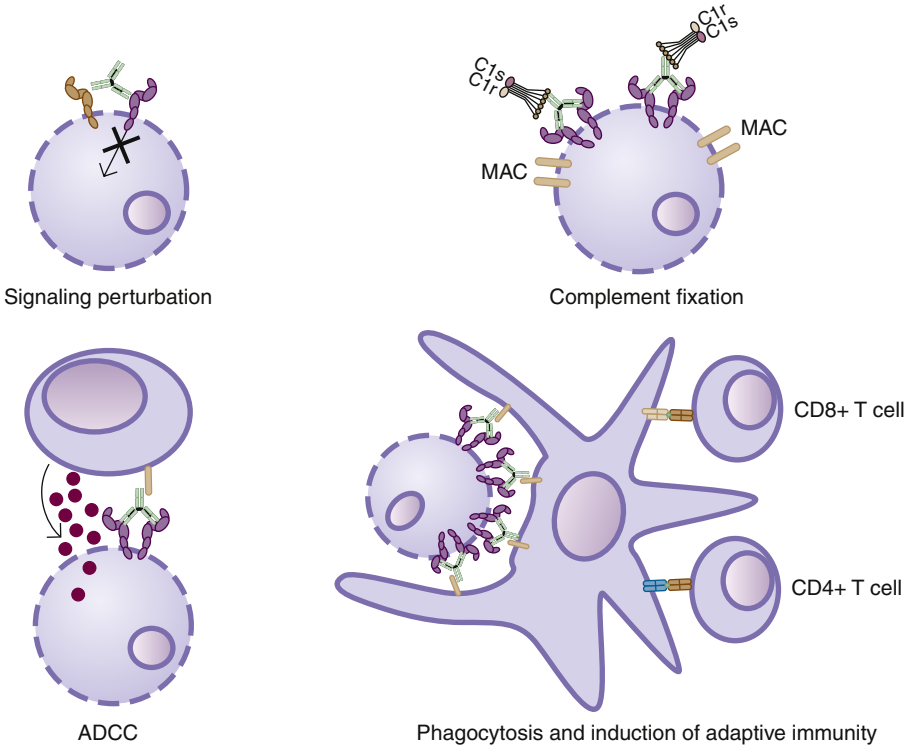


Figure 50-3 Mechanisms of action of monoclonal antibodies. *ADCC*, Antibody-dependent cell-mediated cytotoxicity; *MAC*, membrane attack complex.

allele, which results in reduced serum levels of C1q compared to the C1qG276 allele, had prolonged remission following rituximab monotherapy compared to patients with the G allele.[24] Efforts to optimize CDC led to the development of ofatumumab, which also binds to CD20 but at a distinct epitope from rituximab.[25] Ofatumumab has been demonstrated to induce more CDC in vitro compared to rituximab, even in the setting of low CD20 expression where rituximab is not efficacious.[26] Unlike rituximab, ofatumumab does not directly induce apoptosis in B-cell lines, suggesting that ADCC and CDC are the primary mechanisms of action of ofatumumab.[25] However, preliminary clinical data show that ofatumumab has limited efficacy in the setting of rituximab-resistant follicular lymphoma[27] and comparable efficacy in the setting of non-Hodgkin lymphoma (NHL), although data from trials directly comparing ofatumumab to rituximab are lacking.[25] In the setting of refractory chronic lymphocytic leukemia (CLL), patients demonstrated a 47% to 58% response rate to ofatumumab, leading to its approval by the U.S. Food and Drug Administration (FDA) in 2009 for the treatment of refractory CLL.[28]

Induction of Adaptive Immunity

There is growing evidence to suggest that tumor-targeted antibodies are capable of inducing a therapeutically relevant, tumor-targeted adaptive immune response. ADCC, CDC, and tumor cell signaling perturbation result in target cell apoptosis, which creates tumor cell fragments that can be phagocytosed by antigen-presenting cells (APCs), such as macrophages and dendritic cells. Antibodies can also serve as opsonins by coating tumor cells and engaging Fc receptors on APCs to induce phagocytosis. These engulfed antigens can be processed through the endocytic pathway and presented on MHCII molecules to prime tumor-specific $CD4^+$ T-cell responses. Alternatively, tumor antigens can be loaded on to MHCI molecules to prime $CD8^+$ cytotoxic T cell (CTL) responses, in a process called *cross presentation*[29] (see Figure 50-3). Activated CTLs are capable of directly killing tumor cells that express cognate antigen on MHCI and have powerful prognostic significance in a wide range of human cancers.[30] In vitro, myeloma cells coated with an anti-syndecan1 antibody are capable of activating dendritic cells and inducing the cross presentation of cancer-testis antigen NY-Eso-1, generating NY-Eso-1–specific CTLs capable of lysing myeloma targets.[31] Similarly, antibody-coated melanoma and ovarian cancer cells were capable of inducing cross presentation of various tumor cell antigens, resulting in the generation of melanoma- and ovarian cancer–specific CTLs capable of lysing tumor cell targets.[32] Blockade of inhibitory FcγRIIB enhances dendritic cell activation and cross presentation, suggesting a potential therapeutic approach to boost antibody-initiated adaptive immunity.[33] Recent work in the setting of colorectal cancer shows that cetuximab in combination with chemotherapy induces a potent CTL

Table 50-1 FDA-Approved Antibodies Used in Oncology

Generic Name (Trade Name)	Target	Antibody Isotype	Cancer Indication
Rituximab (Rituxan)	CD20	Chimeric IgG1	Chronic lymphocytic leukemia B-cell non-Hodgkin lymphoma
Trastuzumab (Herceptin)	HER2/neu	Humanized IgG1	Breast cancer, gastric/gastroesophageal junction adenocarcinomas
Alemtuzumab (Campath-1H)	CD52	Humanized IgG1	Chronic lymphocytic leukemia
Cetuximab (Erbitux)	EGF receptor	Chimeric IgG1	Colorectal cancer, squamous carcinoma of the head and neck
Bevacizumab (Avastin)	VEGF	Humanized IgG1	Colorectal cancer, non–small-cell lung cancer, renal cancer, glioblastoma
Panitumumab (Vectibix)	EGF receptor	Human IgG2	Colorectal cancer
Ofatumumab (Arzerra)	CD20	Human IgG1	Chronic lymphocytic leukemia
Pertuzumab (Perjeta)	HER2/neu	Humanized IgG1	Breast cancer
Ipilimumab (Yervoy)	CTLA-4	Human IgG1	Melanoma
Immunoconjugates			
Ibritumomab tiuxetan (Zevalin)	CD20	Murine IgG1	B-cell non-Hodgkin lymphoma
^{131}I-tositumomab/tositumomab (Bexxar)	CD20	Murine IgG2a	B-cell non-Hodgkin lymphoma
Brentuximab vedotin (Adcetris)	CD30	Chimeric IgG1	Anaplastic large-cell lymphoma, Hodgkin's lymphoma

EGF, Epidermal growth factor; *FDA*, U.S. Food and Drug Administration; *VEGF*, vascular endothelial growth factor.

response and thus may contribute to the in vivo activity of cetuximab.[34] Animal models demonstrate that the therapeutic efficacy of HER2/neu-directed antibodies is due in part to induction of an adaptive immune response, as depletion of T cells abrogates antitumor activity.[35,36] Although the preclinical data implicating the induction of adaptive immunity as an important effector mechanism of tumor-targeted antibodies are provocative, more clinical evidence is needed to determine the clinical relevance of this mechanism of action.

Antibodies Targeting Solid Tumors

As mentioned earlier, EGFR family members are overexpressed in a multitude of solid tumors, including colorectal, lung, head and neck, ovarian, and malignant gliomas. The most extensively studied anti-EGFR1 antibody (Table 50-1) is cetuximab, which binds to EGFR1 and blocks ligand binding, receptor dimerization and induces EGFR internalization and degradation, culminating in inhibition of receptor-mediated phosphorylation.[37,38] Preclinical data suggest that ADCC and CDC may be important to the efficacy of cetuximab; however, more clinical data are needed to validate these claims. Cetuximab monotherapy has limited clinical activity but significantly improves outcomes when combined with chemotherapy.[39] First-line therapy with cetuximab added to a regimen of FOLFIRI (leucovorin, fluorouracil, and irinotecan) chemotherapy significantly improves overall survival compared to FOLFIRI alone in the setting of metastatic colorectal cancer.[40] It is important to note that the mutational status of KRAS, which is downstream of EGFR, is critical in predicting response to cetuximab: patients harboring activating KRAS mutations generally do not benefit from cetuximab therapy.[39]

Panitumumab is another antibody targeting EGFR, but unlike cetuximab, panitumumab possesses an IgG2 isotype and thus is unable to induce high levels of ADCC or CDC. Panitumumab significantly improves progression-free survival (PFS) and overall response rates in patients with refractory metastatic colon cancer compared to best supportive care (BSC).[12] Like cetuximab, use of panitumumab is relegated to patients with wild-type KRAS.[41]

Three new anti-EGFR antibodies are currently being evaluated in Phase I and Phase II clinical trials: necitumumab, zalutumumab, and nimotuzumab. Necitumumab is a fully human IgG1 that binds to a similar epitope of EGFR compared to cetuximab and has antitumor activity that is comparable or superior to cetuximab in preclinical models.[42,43] No hypersensitivity reactions have been reported so far, whereas for cetuximab, approximately 3% of patients develop hypersensitivity reactions requiring treatment, mostly during the first infusion of antibody. Phase III studies are ongoing in the setting of non–small-cell lung cancer (NSCLC) (NCT00982111).

Zalutumumab is capable of inducing ADCC in addition to inhibiting receptor phosphorylation. Results from a Phase III trial showed modest improvement in PFS compared to BSC in patients with squamous carcinoma of the head and neck, although no improvement in overall survival was demonstrated.[44]

Nimotuzumab has been shown to sensitize NSCLC cell lines with high EGFR expression to radiation therapy while having no effect on EGFR low-expressing lines.[45] Nimotuzumab has a highly favorable toxicity profile with virtually no grade 3 or 4 skin toxicities observed, often a dose-limiting toxicity seen with other anti-EGFR antibodies. Unlike cetuximab and panitumumab, nimotuzumab has a 10-fold lower affinity for EGFR and binds to EGFR in a manner that still allows the receptor to adopt an active confirmation and mediate ligand independent signaling.[46] This basal level of signaling may be enough to sustain growth of normal cells and explain nimotuzumab's favorable toxicity profile. An alternate hypothesis is that nimotuzumab requires bivalent binding for stable attachment to EGFR, and that normal cells do not have sufficient EGFR expression to mediate enough bivalent binding to be adversely affected.[47] Based on favorable Phase II studies, nimotuzumab is approved for use in more than 20 countries in patients with head and neck cancer and/or gliomas.[48]

HER2/neu is overexpressed and gene amplified in 25% to 30% of breast cancer, and these properties confer a negative prognosis in patients. It is also overexpressed in some adenocarcinomas of the lung, ovary, and gastrointestinal tract.[49] Trastuzumab monotherapy showed a 26% response rate in patients with untreated metastatic breast cancer[50] and significantly improved PFS and OS when combined with chemotherapy, although some patients developed serious cardiotoxicity.[51] The exact mechanism of action of trastuzumab is still unclear but is thought to involve inhibition of receptor dimerization, inhibition of receptor shedding, and activation of immune effector mechanisms such as ADCC.[14]

Trastuzumab emtansine, commonly referred to as trastuzumab-DM1 (T-DM1), is an immunoconjugate in which the cytotoxic agent DM1 is covalently attached to trastuzumab. DM1, a derivative of maytansine, is a highly potent antimitotic but has limited clinical utility due to a poor therapeutic window. Phase I and II studies have demonstrated that targeted delivery of DM1 with T-DM1 significantly improves toxicity and has antitumor activity in patients with metastatic breast cancer previously treated with HER2 targeted agents.[52,53] In addition to the cytotoxic effects of DM1, T-DM1 maintains trastuzumab's mechanisms of action, including signaling perturbation and induction of ADCC.[54] Results from a pivotal Phase III trial demonstrate that T-DM1 improved PFS without significant cardiotoxicity in breast cancer patients whose disease had progressed following trastuzumab therapy.[55]

Pertuzumab is another approved HER2-directed antibody that binds to a distinct epitope from trastuzumab, but has complementary mechanisms of action including inhibition of receptor dimerization and induction of ADCC.[56,57] Results from the CLEOPATRA trial demonstrated that adding pertuzumab to a combination of trastuzumab plus chemotherapy significantly increased PFS in patients with metastatic breast cancer compared to trastuzumab plus chemotherapy alone.[58] Notably, no enhanced cardiotoxicity was seen in patients receiving pertuzumab and trastuzumab combination therapy.[58] These results formed the basis for the FDA approval of pertuzumab in 2012.

Antibodies Targeting Hematological Malignancies

Rituximab is a chimeric antibody that targets CD20 and is used clinically for the treatment of a wide range of B-cell malignancies. A pivotal study leading to FDA approval showed a 48% response rate to rituximab in patients with relapsed low-grade or follicular lymphoma.[59] Combination therapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy improved OS in elderly patients with large B-cell lymphoma.[60] In the setting of CLL, rituximab seems to be more effective in patients who have not received prior chemotherapy.[61] This antibody continues to be an indispensable backbone for the therapy of patients with B-cell malignancies.

Alemtuzumab is approved for the treatment of fludarabine-refractory CLL and binds to CD52, which is highly expressed on various B- and T-cell malignancies. CD52 is a GPI-linked protein with unknown function that is normally expressed in parts of the male reproductive tract and on a wide range of leukocytes, including lymphocytes and monocytes. The exact mechanism of action of alemtuzumab is unclear but is thought to involve CDC, ADCC, and induction of apoptosis. In addition to chemoresistant CLL, alemtuzumab has activity in the setting of prolymphocytic leukemia (PLL) and low-grade NHL.[62-64] Significant toxicities attributed to alemtuzumab therapy include myelosuppression and opportunistic infections, sometimes leading to death. These toxicities are presumably a result of CD52 expression on normal leukocytes.

CD30 is a member of the TNF superfamily and is expressed by the malignant Reed-Sternberg cells characteristic of Hodgkin's lymphoma. Brentuximab vedotin is an antibody-drug conjugate composed of the anti-CD30 antibody brentuximab linked to the anti-microtubule agent

monomethyl auristatin E (MMAE). On binding to CD30, the antibody-CD30 complex is internalized and trafficked to the lysosome, where MMAE is released by proteolysis. MMAE binds to tubulin, resulting in cell cycle arrest and apoptosis. Brentuximab vedotin is generally well tolerated, with neutropenia and peripheral neuropathy being the most common serious adverse events. Results of Phase II studies show a 75% overall response rate in patients with relapsed or refractory Hodgkin's lymphoma after stem cell transplant and an 86% response rate in patients with anaplastic large-cell lymphoma.[65,66]

CD74 is another interesting target for non–CD20-expressing malignancies such as multiple myeloma. CD74 was originally described as the invariant chain of MHC Class II and was shown to be critical for proper loading of peptide and trafficking of MHCII molecules to the cell surface. More recently, non-MHC Class II–related functions of CD74 have been described, including its role in cell survival and proliferation. The ligand for CD74 is macrophage inhibitory factor (MIF), and because CD74 is not capable of initiating signaling on its own, CD44 is required as a co-receptor. CD74 is expressed on 85% of NHL, CLL, and various multiple myeloma cell lines and has limited cell surface expression on normal cells.[67] Milatuzumab is a chimeric antibody that targets CD74 and has shown efficacy in preclinical studies of multiple myeloma and NHL.[68,69] Milatuzumab does not induce ADCC or CDC and is rapidly internalized on binding to CD74. This rapid internalization of antibody-CD74 complexes makes milatuzumab a prime candidate for the development of an antibody drug conjugate. Preclinical studies on a milatuzumab-doxorubicin conjugate are encouraging.[70] Currently, milatuzumab is in multiple Phase I and II studies for multiple myeloma (NCT00421525), CLL (NCT00603668), and NHL (NCT00989586).

Antibodies Targeting Immune Cells

Ipilimumab, an antibody that targets cytotoxic T-lymphocyte–associated antigen (CTLA-4), is the first anti-cancer antibody to gain FDA approval that specifically targets non-malignant immune cells. CTLA-4 is expressed on T-cells and is a negative regulator of T-cell activation (Figure 50-4). T-cell activation is initiated by engagement of the T-cell receptor (TCR) with peptide-loaded MHC; however, co-stimulatory signals are needed to ensure proper activation. The most important of these signals is the interaction of CD28 on T cells with the B7 family members CD80 and CD86 on APCs. CTLA-4 binds to CD80 and CD86 with higher affinity than CD28 and serves to inhibit activation by outcompeting CD28 and transducing inhibitory signals. CTLA-4 suppresses antitumor immunity by downregulating $CD4^+$ T-cell responses and enhancing the immunosuppressive effect of T-regulatory cells (Treg).[71] Targeting CTLA-4 with a monoclonal antibody demonstrated remarkable antitumor activity in preclinical models,[72] and ipilimumab showed promise in early clinical studies that demonstrated antitumor activity and activation of antitumor immune responses.[73] Notable adverse events included immune-related diarrhea and vitiligo, consistent with ipilimumab's mechanism of action. A pivotal Phase III trial showed that ipilimumab therapy improved median survival by 3.6 months in patients with metastatic melanoma, making ipilimumab the only therapeutic demonstrated to improve survival in patients with metastatic melanoma.[74]

Another emerging target for antibody therapy is programmed cell death protein 1 (PD-1). PD-1 is upregulated on activated T cells and serves to dampen effector T-cell responses in peripheral tissues, including the tumor microenvironment. PD-1 is also expressed on Treg cells and has been demonstrated to promote the development and activity of peripherally induced Treg cells.[75] PD-1 ligands, most notably PD-L1, are expressed on tumor cells and myeloid cells in the tumor microenvironment, providing further rationale for therapeutic targeting of this pathway.[76] Data from two large Phase I trials of the anti-PD-1 antibody BMS-936558, a fully human IgG4, demonstrated safety and antitumor efficacy in the setting of metastatic melanoma, renal cancer, and NSCLC.[77,78] Remarkably, monotherapy with BMS-936558 resulted in durable antitumor responses, even in the setting of NSCLC, which in contrast to melanoma and renal cancer had not been considered to be an immunotherapy-responsive tumor. In addition to multiple Phase I and II trials, BMS-936558 is also being evaluated in combination with ipilimumab for the treatment of metastatic melanoma (NCT01024231).

Whereas ipilimumab and BMS-936558 block signaling through inhibitory receptors, another approach to re-activate anti-tumor immunity is to activate stimulatory receptors. CD40 is a stimulatory receptor expressed by many different cell types including B cells, dendritic cells, monocytes, endothelial cells, and fibroblasts. The ligand for CD40, CD40L, is primarily expressed on activated T cells and platelets. Engagement of CD40 leads to B-cell proliferation and maturation of dendritic cells, resulting in enhancement of T-cell activation. Interestingly, CD40 is expressed on a wide range of malignancies, including nearly all B-cell malignancies and many solid tumors, and engagement of CD40 on some malignant cells leads to apoptosis.[79] Three antibodies targeting CD40 are currently being evaluated for safety and efficacy. Dacetuzumab (SGN-40) is a humanized IgG1 with weak agonist activity that is capable of inducing ADCC in vitro and has antitumor activity against B-cell

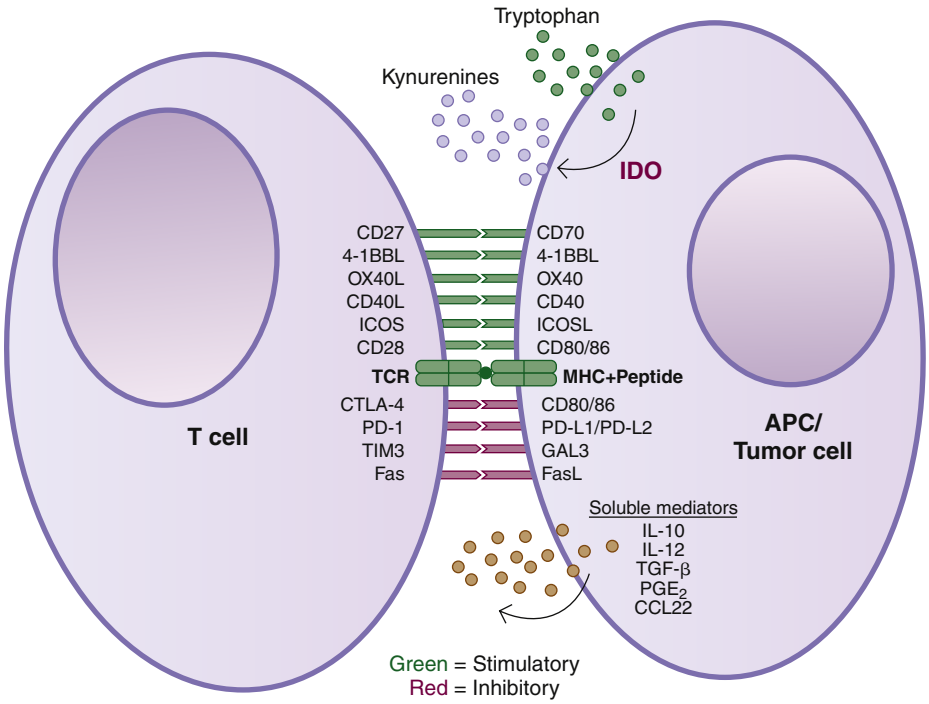


Figure 50-4 Potential targets to enhance T-cell activation. *APC*, Antigen-presenting cell; *IDO*, indoleamine 2,3-dioxygenase; *MHC*, major histocompatibility complex; *TCR*, T-cell receptor.

lymphoma xenografts in vivo.[80] A Phase I study of dacetuzumab showed encouraging antitumor activity in NHL patients, with the most common adverse events being related to cytokine release syndrome, including fever, headache, and fatigue.[81]

Lucatumumab (HCD122) is an IgG1 that is a pure antagonist of CD40 capable of inducing ADCC and has shown activity against B-cell CLL in preclinical models.[82] A recent Phase I study showed that lucatumumab is reasonably well tolerated; however, it shows minimal activity as a single agent in the setting of relapsed CLL.[83]

CP-870,813 is a fully human IgG2 that functions as a CD40 agonist and has antitumor and immunostimulatory activity in patients with melanoma.[84] Remarkably, CP-870,813 in combination with gemcitabine has also demonstrated activity in a small cohort of patients with pancreatic cancer.[85] In the same study, the authors used a mouse model of spontaneous pancreatic cancer to identify a potential mechanism of action of CP-870,813. Unexpectedly, the antitumor activity of CP-870,813 was independent of T cells and dependent on its capacity to alter the phenotype of tumor-associated macrophages to a more tumoricidal phenotype.[85]

Antibodies Targeting Angiogenesis

The tumor stroma is enriched with pro-angiogenic factors that facilitate recruitment and remodeling of blood vessels essential for tumor growth. A critical driver of vascular endothelial cell migration and proliferation is the vascular endothelial growth factor (VEGF) family, comprising six members (VEGF-A through -E and placental growth factor [PlGF]). VEGF has three known receptors, VEGFR1 through -3, with VEGFR-1 and -2 being most relevant to tumor biology. VEGF levels are increased in a wide range of malignancies and are often associated with a poor prognosis.[86] Bevacizumab is an antibody targeting VEGF-A and has been shown to improve survival when combined with chemotherapy in patients with metastatic colon cancer.[87] The most frequent adverse event was hypertension, with no increased incidence of hemorrhage, frequently observed in other trials of bevacizumab.[87] The addition of bevacizumab to a regimen of carboplatin and paclitaxel improved response rates and survival in patients with NSCLC, although a significant increase in bleeding, specifically lethal pulmonary hemorrhage, was observed.[88] Single-agent bevacizumab received accelerated approval for the treatment of glioblastoma multiforme (GBM) based on demonstrated durable response rates and manageable adverse events when administered as monotherapy.[89,90] Larger clinical trials are needed to evaluate bevacizumab's impact on the clinical course of GBM. The role of bevacizumab in the setting of HER2/neu–negative metastatic breast cancer is a subject of debate. In 2008, bevacizumab received accelerated approval for the treatment of HER2/neu–negative metastatic breast cancer based largely on results from the E2100 trial, which demonstrated that combination therapy with bevacizumab and paclitaxel improved PFS compared to paclitaxel alone,

with no significant difference in OS between the two treatment arms.[91] However, subsequent trials showed that the addition of bevacizumab to chemotherapy resulted in only modest improvements in PFS, with no impact on overall survival.[92-94] Based on the results of these trials, the FDA revoked approval of bevacizumab for the treatment of metastatic breast cancer in 2011, citing an unfavorable risk/benefit profile. Debate continues on the utility of using PFS or pathological complete response (pCR) versus overall survival to evaluate the clinical efficacy of new drugs and treatment regimens.

VEGFR-2 is upregulated in the tumor vasculature. Signaling through VEGFR-2, as opposed to VEGF-1, is sufficient to mediate the pro-angiogenic functions of VEGF.[95] The VEGF/VEGFR-2 axis is also detrimental to the antitumor immune response, whereas signaling through VEGF-1 may promote lymphoid development.[96] Ramucirumab is a fully human IgG1 that specifically targets VEGFR-2 and inhibits VEGF binding. A Phase I study demonstrated antitumor efficacy. However, significant toxicities were observed including hypertension, abdominal pain, vomiting, and deep-vein thromboses.[97] Several Phase II and III studies in the setting of renal, breast, colon, and NSCLC are ongoing.[95]

Recent work suggests that cancer-associated fibroblasts (CAFs) are an important source of pro-angiogenic stimuli, such as VEGF and fibroblast growth factor (FGF). CAFs from tumors resistant to anti-VEGF therapy upregulate expression of other pro-angiogenic factors and may contribute to resistance of VEGF targeted therapy.[98] A potential therapeutic target for CAFs is fibroblast activated protein-alpha (FAP), which is highly expressed on CAFs compared to normal fibroblasts.[99] Depletion of FAP$^+$ stromal cells results in inhibition of tumor growth and activation of antitumor immune responses.[100] However, clinical efforts to target FAP using the anti-FAP antibody sibrotuzumab have been disappointing.[101] One possible explanation for this lack of efficacy is that sibrotuzumab does not inhibit the enzymatic activity of FAP.[102] Conjugation of sibrituzumab to the antimitotic DM1 has shown promise in a preclinical study. However, further studies are needed to determine the clinical utility of this antibody-drug conjugate.[103]

Antibody Engineering

Advancements in protein engineering have allowed for the modification of antibody structure in an attempt to improve targeting and enhance antitumor activity. Bispecific antibodies represent an important class of engineered antibodies that are capable of recognizing two distinct antigens. The most common bispecific antibodies target a tumor antigen and CD3, which is a component of the T-cell receptor. Bispecific T-cell engagers (BiTEs) are a class of bispecific antibodies that link the variable domains of an antitumor antibody with the variable region of an anti-CD3 antibody. BiTEs induce robust polyclonal T-cell activation, resulting in targeted release of perforin and granzyme and tumor cell apoptosis. Importantly, BiTES are capable of stimulating T-cells and inducing tumor cell lysis in the absence of co-stimulatory signals and MHC expression. Blinatumomab is a BiTE that binds to CD19 and CD3 that has clinical activity in patients with minimal residual disease (MRD) positive, B-cell acute lymphoblastic leukemia (ALL).[104] Of the 20 patients treated, 16 became MRD negative, and 12 of the 16 responders had never turned MRD negative with any previous treatment.[104]

Bispecific antibodies that have an intact Fc domain are termed *trifunctional antibodies*, because of their capacity to bind and activate FcγR$^+$ immune effectors. Trifunctional antibodies targeting a tumor antigen and CD3 are capable of inducing T-cell–mediated lysis in addition to ADCC and phagocytosis. Catumaxomab is an example of a trifunctional antibody that has dual specificity for CD3 and epithelial cell adhesion molecule (EpCAM), which is ubiquitously expressed on tumors of epithelial origin.[105] Catumaxomab contains one light chain and one heavy chain from an anti-EpCAM mouse IgG2, and one light chain and one heavy chain from an anti-CD3 rat IgG2b. The Fc domain generated from mouse and rat constant regions binds preferentially to activating FcγRs, with low binding to FcγRIIB, resulting in potent ADCC and phagocytosis of EpCAM$^+$ tumor cells.[105] Interestingly, preclinical studies demonstrated that the related antibody, BiLU (anti-human EpCAM × anti-mouse CD3), was capable of eliciting a protective adaptive immune response against syngeneic tumors.[106] A small clinical study showed that catumaxomab was capable of eliciting tumor-specific T cells, suggesting that induction of adaptive immunity may contribute to catumaxomab's mechanisms of action.[107] Catumaxomab is approved in the European Union for the treatment of malignant ascites and been shown to improve quality of life and increase time to next paracentesis.[108] The main adverse events associated with catumaxomab are related to severe cytokine release syndrome, necessitating careful dosing and patient monitoring.

Modifications to the Fc domain are capable of enhancing effector functions and improving the pharmacokinetic profile of monoclonal antibodies. Fc mutational analyses have identified optimal amino acid sequences that result in greater binding to activating FcγRs and C1q to enhance ADCC and CDC. An anti-CD19 antibody engineered to have greater FcγR binding demonstrated more potent ADCC, phagocytosis, and antitumor activity compared to its parent antibody.[109] Improving interactions with neonatal

FcRs can extend serum-half life and potentially reduce the cost of treatment.[110] However, modulation of oligosaccharide residues within the CH2 domain is the modification that has been most widely used to date.

Human IgG molecules contain two N-linked, complex-type oligosaccharides within the CH2 domain. These oligosaccharides contain a fucose residue, and removal of this residue dramatically enhances the capacity of antibodies to mediate ADCC, even at low antigen densities.[111] Obinutuzumab is a defucosylated anti-CD20 antibody that displays greater ADCC in vitro and anti-lymphoma activity in vivo compared to rituximab.[112] Two Phase I studies of obinutuzumab demonstrated activity in the setting of relapsed NHL with manageable adverse events, mostly associated with infusion-related toxicity.[113,114] Interestingly, both studies showed activity (13% to 22% response rate) in a subset of patients refractory to rituximab.[113,114]

Summary and Future Directions

Monoclonal antibodies have revolutionized the treatment of cancer and will continue to serve as mainstays of cancer therapy for the foreseeable future. Antibodies offer the unique capability to specifically engage a tumor cell and stimulate a multifaceted program of cell death involving perturbation of homeostatic signaling and activation of the host immune system, without overt toxicity. Advances in antibody therapy are contingent on the identification and validation of new tumor antigens, manipulation of the tumor microenvironment, and optimization of antibody structure to improve effector functions and cytotoxic drug delivery.

Key References

1. Glanville J, Zhai W, Berka J, et al. Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proc Natl Acad Sci U S A*. 2009;106:20216-20221.
2. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol*. 2007;7:715-725.
5. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol*. 2009;157:220-233.
6. Mascelli MA, Zhou H, Sweet R, et al. Molecular, biologic, and pharmacokinetic properties of monoclonal antibodies: impact of these parameters on early clinical development. *J Clin Pharmacol*. 2007;47:553-565.
10. Hoogenboom HR. Selecting and screening recombinant antibody libraries. *Nat Biotechnol*. 2005;23:1105-1116.
11. Lonberg N. Human antibodies from transgenic animals. *Nat Biotechnol*. 2005;23:1117-1125.
12. Van Cutsem E, Peeters M, Siena S, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol*. 2007;25:1658-1664.
14. Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. *N Engl J Med*. 2007;357:39-51.
17. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol*. 2003;21:3940-3947.
18. Bibeau F, Lopez-Crapez E, Di Fiore F, et al. Impact of FcγRIIa-FcγRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol*. 2009;27:1122-1129.
19. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008;26:1789-1796.
22. Cragg MS, Morgan SM, Chan HT, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood*. 2003;101:1045-1052.
23. Di Gaetano N, Cittera E, Nota R, et al. Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol*. 2003;171:1581-1587.
24. Racila E, Link BK, Weng WK, et al. A polymorphism in the complement component C1qA correlates with prolonged response following rituximab therapy of follicular lymphoma. *Clin Cancer Res*. 2008;14:6697-6703.
25. Cheson BD. Ofatumumab, a novel anti-CD20 monoclonal antibody for the treatment of B-cell malignancies. *J Clin Oncol*. 2010;28:3525-3530.
26. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood*. 2004;104:1793-1800.
27. Czuczman MS, Fayad L, Delwail V, et al. Ofatumumab monotherapy in rituximab-refractory follicular lymphoma: results from a multicenter study. *Blood*. 2012;119:3698-3704.
28. Wierda WG, Kipps TJ, Mayer J, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. *J Clin Oncol*. 2010;28:1749-1755.
30. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12:298-306.
32. Cioca DP, Deak E, Cioca F, Paunescu V. Monoclonal antibodies targeted against melanoma and ovarian tumors enhance dendritic cell-mediated cross-presentation of tumor-associated antigens and efficiently cross-prime CD8+ T cells. *J Immunother*. 2006;29:41-52.
33. Dhodapkar KM, Kaufman JL, Ehlers M, et al. Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A*. 2005;102:2910-2915.
34. Correale P, Botta C, Cusi M, et al. Cetuximab ± chemotherapy enhances dendritic cell-mediated phagocytosis of colon cancer cells and ignites a highly efficient colon cancer antigen-specific cytotoxic T-cell response in vitro. *Int J Cancer*. 2012;130:1577-1589.
35. Park S, Jiang Z, Mortenson ED, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell*. 2010;18:160-170.
36. Wang S, Astsaturov IA, Bingham CA, et al. Effective antibody therapy induces host-protective antitumor immunity that is augmented by TLR4 agonist treatment. *Cancer Immunol Immunother*. 2012;61:49-61.
37. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell*. 2005;7:301-311.
38. Doody JF, Wang Y, Patel SN, et al. Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non small cell lung cancers. *Mol Cancer Ther*. 2007;6:2642-2651.
39. Van Cutsem E, Kohne CH, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med*. 2009;360:1408-1417.

40. Van Cutsem E, Kohne CH, Lang I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol*. 2011;29:2011-2019.
41. Amado RG, Wolf M, Peeters M, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008;26:1626-1634.
42. Li S, Kussie P, Ferguson KM. Structural basis for EGF receptor inhibition by the therapeutic antibody IMC-11F8. *Structure*. 2008;16:216-227.
43. Kuenen B, Witteveen PO, Ruijter R, et al. A phase I pharmacologic study of necitumumab (IMC-11F8), a fully human IgG1 monoclonal antibody directed against EGFR in patients with advanced solid malignancies. *Clin Cancer Res*. 2010;16:1915-1923.
44. Machiels JP, Subramanian S, Ruzsa A, et al. Zalutumumab plus best supportive care versus best supportive care alone in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy: an open-label, randomised phase 3 trial. *Lancet Oncol*. 2011;12:333-343.
45. Akashi Y, Okamoto I, Iwasa T, et al. Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status. *Br J Cancer*. 2008;98:749-755.
46. Talavera A, Friemann R, Gomez-Puerta S, et al. Nimotuzumab, an antitumor antibody that targets the epidermal growth factor receptor, blocks ligand binding while permitting the active receptor conformation. *Cancer Res*. 2009;69:5851-5859.
47. Garrido G, Tikhomirov IA, Rabasa A, et al. Bivalent binding by intermediate affinity of nimotuzumab: a contribution to explain antibody clinical profile. *Cancer Biol Ther*. 2011;11:373-382.
48. Boland W, Bebb G. The emerging role of nimotuzumab in the treatment of non-small cell lung cancer. *Biologics*. 2010;4:289-298.
49. Chen JS, Lan K, Hung MC. Strategies to target HER2/neu overexpression for cancer therapy. *Drug Resist Updat*. 2003;6:129-136.
52. Krop IE, Beeram M, Modi S, et al. Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol*. 2010;28:2698-2704.
53. Krop IE, Lorusso P, Miller KD, et al. A phase II study of trastuzumab emtansine in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer who were previously treated with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine. *J Clin Oncol*. 2012;30:3234-3241.
54. Junttila TT, Li G, Parsons K, Phillips GL, Sliwkowski MX. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat*. 2011;128:347-356.
55. Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med*. 2012;367:1783-1791.
56. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*. 2004;5:317-328.
57. Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, Hasmann M. Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Res*. 2009;69:9330-9336.
58. Baselga J, Cortes J, Kim SB, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med*. 2012;366:109-119.
61. Mavromatis B, Cheson BD. Monoclonal antibody therapy of chronic lymphocytic leukemia. *J Clin Oncol*. 2003;21:1874-1881.
65. Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. *J Clin Oncol*. 2012;30:2183-2189.
66. Pro B, Advani R, Brice P, et al. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study. *J Clin Oncol*. 2012;30:2190-2196.
67. Berkova Z, Tao RH, Samaniego F. Milatuzumab—a promising new immunotherapeutic agent. *Expert Opin Investig Drugs*. 2010;19:141-149.
68. Stein R, Qu Z, Cardillo TM, et al. Antiproliferative activity of a humanized anti-CD74 monoclonal antibody, hLL1, on B-cell malignancies. *Blood*. 2004;104:3705-3711.
69. Stein R, Smith MR, Chen S, Zalath M, Goldenberg DM. Combining milatuzumab with bortezomib, doxorubicin, or dexamethasone improves responses in multiple myeloma cell lines. *Clin Cancer Res*. 2009;15:2808-2817.
70. Sapra P, Stein R, Pickett J, et al. Anti-CD74 antibody-doxorubicin conjugate, IMMU-110, in a human multiple myeloma xenograft and in monkeys. *Clin Cancer Res*. 2005;11:5257-5264.
71. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12:252-264.
73. Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 2003;100:4712-4717.
74. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
75. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.
77. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
78. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
79. Vonderheide RH. Prospect of targeting the CD40 pathway for cancer therapy. *Clin Cancer Res*. 2007;13:1083-1088.
80. Law CL, Gordon KA, Collier J, et al. Preclinical antilymphoma activity of a humanized anti-CD40 monoclonal antibody, SGN-40. *Cancer Res*. 2005;65:8331-8338.
81. Advani R, Forero-Torres A, Furman RR, et al. Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma. *J Clin Oncol*. 2009;27:4371-4377.
82. Luqman M, Klabunde S, Lin K, et al. The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. *Blood*. 2008;112:711-720.
83. Byrd JC, Kipps TJ, Flinn IW, et al. Phase I study of the anti-CD40 humanized monoclonal antibody lucatumumab (HCD122) in relapsed chronic lymphocytic leukemia. *Leuk Lymphoma*. 2012;53:2136-2142.
84. Vonderheide RH, Flaherty KT, Khalil M, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol*. 2007;25:876-883.
85. Beatty GL, Chiorean EG, Fishman MP, et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. 2011;331:1612-1616.
87. Ohm JE, Gabrilovich DI, Sempowski GD, et al. VEGF inhibits T cell development and may contribute to tumor-induced immune suppression. *Blood*. 2003;101:4878-4886.
88. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med*. 2006;355:2542-2550.
89. Kreisl TN, Kim L, Moore K, et al. Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *J Clin Oncol*. 2009;27:740-745.
90. Vredenburgh JJ, Desjardins A, Herndon 2nd JE, et al. Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. *J Clin Oncol*. 2007;25:4722-4729.
91. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med*. 2007;357:2666-2676.
92. Robert NJ, Dieras V, Glaspy J, et al. RIBBON-1: randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2-negative, locally recurrent or metastatic breast cancer. *J Clin Oncol*. 2011;29:1252-1260.
93. Brufsky AM, Hurvitz S, Perez E, et al. RIBBON-2: a randomized, double-blind, placebo-controlled, phase III trial evaluating the efficacy and safety of bevacizumab in combination with chemotherapy for second-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. *J Clin Oncol*. 2011;29:4286-4293.
94. Pivot X, Schneeweiss A, Verma S, et al. Efficacy and safety of bevacizumab in combination with docetaxel for the first-line treatment of elderly patients with locally recurrent or metastatic breast cancer: results from AVADO. *Eur J Cancer*. 2011;47:2387-2395.

95. Spratlin J. Ramucirumab (IMC-1121B): Monoclonal antibody inhibition of vascular endothelial growth factor receptor-2. *Curr Oncol Rep*. 2011;13:97-102.
96. Huang Y, Chen X, Dikov MM, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood*. 2007;110:624-631.
97. Spratlin JL, Cohen RB, Eadens M, et al. Phase I pharmacologic and biologic study of ramucirumab (IMC-1121B), a fully human immunoglobulin G1 monoclonal antibody targeting the vascular endothelial growth factor receptor-2. *J Clin Oncol*. 2010;28:780-787.
98. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer*. 2010;10:505-514.
99. Xing F, Saidou J, Watabe K. Cancer Associated Fibroblasts (CAFs) in tumor microenvironment. *Front Biosci*. 2010;15:166-179.
100. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*. 2010;330:827-830.
101. Hofheinz RD, al-Batran SE, Hartmann F, et al. Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibrotuzumab in patients with metastatic colorectal cancer. *Onkologie*. 2003;26:44-48.
102. Santos AM, Jung J, Aziz N, Kissil JL, Pure E. Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J Clin Invest*. 2009;119:3613-3625.
103. Ostermann E, Garin-Chesa P, Heider KH, et al. Effective immunoconjugate therapy in cancer models targeting a serine protease of tumor fibroblasts. *Clin Cancer Res*. 2008;14:4584-4592.
104. Topp MS, Kufer P, Gokbuget N, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol*. 2011;29:2493-2498.
105. Seimetz D, Lindhofer H, Bokemeyer C. Development and approval of the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3) as a targeted cancer immunotherapy. *Cancer Treat Rev*. 2010;36:458-467.
107. Strohlein MA, Siegel R, Jager M, Lindhofer H, Jauch KW, Heiss MM. Induction of anti-tumor immunity by trifunctional antibodies in patients with peritoneal carcinomatosis. *J Exp Clin Cancer Res*. 2009;28:18.
108. Jager M, Schoberth A, Ruf P, et al. Immunomonitoring results of a phase II/III study of malignant ascites patients treated with the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3). *Cancer Res*. 2012;72:24-32.
109. Horton HM, Bernett MJ, Pong E, et al. Potent in vitro and in vivo activity of an Fc-engineered anti-CD19 monoclonal antibody against lymphoma and leukemia. *Cancer Res*. 2008;68:8049-8057.
110. Gong R, Wang Y, Feng Y, Zhao Q, Dimitrov DS. Shortened engineered human antibody CH2 domains: increased stability and binding to the human neonatal Fc receptor. *J Biol Chem*. 2011;286:27288-27293.
111. Niwa R, Sakurada M, Kobayashi Y, et al. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. *Clin Cancer Res*. 2005;11:2327-2336.
112. Mossner E, Brunker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood*. 2010;115:4393-4402.
113. Salles G, Morschhauser F, Lamy T, et al. Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. *Blood*. 2012;119:5126-5132.
114. Sehn LH, Assouline SE, Stewart DA, et al. A phase 1 study of obinutuzumab induction followed by 2 years of maintenance in patients with relapsed CD20-positive B-cell malignancies. *Blood*. 2012;119:5118-5125.

References

1. Glanville J, Zhai W, Berka J, et al. Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proc Natl Acad Sci U S A*. 2009;106:20216-20221.
2. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol*. 2007;7:715-725.
3. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975;256:495-497.
4. Tjandra JJ, Ramadi L, McKenzie IF. Development of human anti-murine antibody (HAMA) response in patients. *Immunol Cell Biol*. 1990;68:367-376.
5. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol*. 2009;157:220-233.
6. Mascelli MA, Zhou H, Sweet R, et al. Molecular, biologic, and pharmacokinetic properties of monoclonal antibodies: impact of these parameters on early clinical development. *J Clin Pharmacol*. 2007;47:553-565.
7. Neuberger MS. Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. *EMBO J*. 1983;2:1373-1378.
8. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci U S A*. 1984;81:6851-6855.
9. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature*. 1986;321:522-525.
10. Hoogenboom HR. Selecting and screening recombinant antibody libraries. *Nat Biotechnol*. 2005;23:1105-1116.
11. Lonberg N. Human antibodies from transgenic animals. *Nat Biotechnol*. 2005;23:1117-1125.
12. Van Cutsem E, Peeters M, Siena S, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol*. 2007;25:1658-1664.
13. Yarden Y. The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. *Eur J Cancer*. 2001;37(suppl 4):S3-S8.
14. Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. *N Engl J Med*. 2007;357:39-51.
15. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med*. 2000;6:443-446.
16. Cartron G, Dacheux L, Salles G, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood*. 2002;99:754-758.
17. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol*. 2003;21:3940-3947.
18. Bibeau F, Lopez-Crapez E, Di Fiore F, et al. Impact of FcγRIIa-FcγRIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol*. 2009;27:1122-1129.
19. Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008;26:1789-1796.
20. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood*. 1997;90:1109-1114.
21. Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J Biol Chem*. 2001;276:6591-6604.
22. Cragg MS, Morgan SM, Chan HT, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood*. 2003;101:1045-1052.
23. Di Gaetano N, Cittera E, Nota R, et al. Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol*. 2003;171:1581-1587.
24. Racila E, Link BK, Weng WK, et al. A polymorphism in the complement component C1qA correlates with prolonged response following rituximab therapy of follicular lymphoma. *Clin Cancer Res*. 2008;14:6697-6703.
25. Cheson BD. Ofatumumab, a novel anti-CD20 monoclonal antibody for the treatment of B-cell malignancies. *J Clin Oncol*. 2010;28:3525-3530.
26. Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood*. 2004;104:1793-1800.
27. Czuczman MS, Fayad L, Delwail V, et al. Ofatumumab monotherapy in rituximab-refractory follicular lymphoma: results from a multicenter study. *Blood*. 2012;119:3698-3704.
28. Wierda WG, Kipps TJ, Mayer J, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. *J Clin Oncol*. 2010;28:1749-1755.
29. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature*. 1998;392:86-89.
30. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12:298-306.
31. Dhodapkar KM, Krasovsky J, Williamson B, Dhodapkar MV. Antitumor monoclonal antibodies enhance cross-presentation of cellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. *J Exp Med*. 2002;195:125-133.
32. Cioca DP, Deak E, Cioca F, Paunescu V. Monoclonal antibodies targeted against melanoma and ovarian tumors enhance dendritic cell-mediated cross-presentation of tumor-associated antigens and efficiently cross-prime CD8+ T cells. *J Immunother*. 2006;29:41-52.
33. Dhodapkar KM, Kaufman JL, Ehlers M, et al. Selective blockade of inhibitory Fcgamma receptor enables human dendritic cell maturation with IL-12p70 production and immunity to antibody-coated tumor cells. *Proc Natl Acad Sci U S A*. 2005;102:2910-2915.
34. Correale P, Botta C, Cusi M, et al. Cetuximab ± chemotherapy enhances dendritic cell-mediated phagocytosis of colon cancer cells and ignites a highly efficient colon cancer antigen-specific cytotoxic T-cell response in vitro. *Int J Cancer*. 2012;130:1577-1589.
35. Park S, Jiang Z, Mortenson ED, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell*. 2010;18:160-170.
36. Wang S, Astsaturov IA, Bingham CA, et al. Effective antibody therapy induces host-protective antitumor immunity that is augmented by TLR4 agonist treatment. *Cancer Immunol Immunother*. 2012;61:49-61.
37. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell*. 2005;7:301-311.
38. Doody JF, Wang Y, Patel SN, et al. Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non small cell lung cancers. *Mol Cancer Ther*. 2007;6:2642-2651.
39. Van Cutsem E, Kohne CH, Hitre E, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med*. 2009;360:1408-1417.
40. Van Cutsem E, Kohne CH, Lang I, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol*. 2011;29:2011-2019.
41. Amado RG, Wolf M, Peeters M, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008;26:1626-1634.
42. Li S, Kussie P, Ferguson KM. Structural basis for EGF receptor inhibition by the therapeutic antibody IMC-11F8. *Structure*. 2008;16:216-227.
43. Kuenen B, Witteveen PO, Ruijter R, et al. A phase I pharmacologic study of necitumumab (IMC-11F8), a fully human IgG1 monoclonal antibody directed against EGFR in patients with advanced solid malignancies. *Clin Cancer Res*. 2010;16:1915-1923.
44. Machiels JP, Subramanian S, Ruzsa A, et al. Zalutumumab plus best supportive care versus best supportive care alone in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck after failure of platinum-based chemotherapy: an open-label, randomised phase 3 trial. *Lancet Oncol*. 2011;12:333-343.

45. Akashi Y, Okamoto I, Iwasa T, et al. Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status. *Br J Cancer*. 2008;98:749-755.
46. Talavera A, Friemann R, Gomez-Puerta S, et al. Nimotuzumab, an antitumor antibody that targets the epidermal growth factor receptor, blocks ligand binding while permitting the active receptor conformation. *Cancer Res*. 2009;69:5851-5859.
47. Garrido G, Tikhomirov IA, Rabasa A, et al. Bivalent binding by intermediate affinity of nimotuzumab: a contribution to explain antibody clinical profile. *Cancer Biol Ther*. 2011;11:373-382.
48. Boland W, Bebb G. The emerging role of nimotuzumab in the treatment of non-small cell lung cancer. *Biologics*. 2010;4:289-298.
49. Chen JS, Lan K, Hung MC. Strategies to target HER2/neu overexpression for cancer therapy. *Drug Resist Updat*. 2003;6:129-136.
50. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2002;20:719-726.
51. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
52. Krop IE, Beeram M, Modi S, et al. Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol*. 2010;28:2698-2704.
53. Krop IE, Lorusso P, Miller KD, et al. A phase II study of trastuzumab emtansine in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer who were previously treated with trastuzumab, lapatinib, an anthracycline, a taxane, and capecitabine. *J Clin Oncol*. 2012;30:3234-3241.
54. Junttila TT, Li G, Parsons K, Phillips GL, Sliwkowski MX. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat*. 2011;128:347-356.
55. Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med*. 2012;367:1783-1791.
56. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell*. 2004;5:317-328.
57. Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, Hasmann M. Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Res*. 2009;69:9330-9336.
58. Baselga J, Cortes J, Kim SB, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med*. 2012;366:109-119.
59. McLaughlin P, Grillo-Lopez AJ, Link BK, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol*. 1998;16:2825-2833.
60. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med*. 2002;346:235-242.
61. Mavromatis B, Cheson BD. Monoclonal antibody therapy of chronic lymphocytic leukemia. *J Clin Oncol*. 2003;21:1874-1881.
62. Osterborg A, Dyer MJ, Bunjes D, et al. Phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. European Study Group of CAMPATH-1H Treatment in Chronic Lymphocytic Leukemia. *J Clin Oncol*. 1997;15:1567-1574.
63. Pawson R, Dyer MJ, Barge R, et al. Treatment of T-cell prolymphocytic leukemia with human CD52 antibody. *J Clin Oncol*. 1997;15:2667-2672.
64. Lundin J, Osterborg A, Brittinger G, et al. CAMPATH-1H monoclonal antibody in therapy for previously treated low-grade non-Hodgkin's lymphomas: a phase II multicenter study. European Study Group of CAMPATH-1H Treatment in Low-Grade Non-Hodgkin's Lymphoma. *J Clin Oncol*. 1998;16:3257-3263.
65. Younes A, Gopal AK, Smith SE, et al. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. *J Clin Oncol*. 2012;30:2183-2189.
66. Pro B, Advani R, Brice P, et al. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study. *J Clin Oncol*. 2012;30:2190-2196.
67. Berkova Z, Tao RH, Samaniego F. Milatuzumab—a promising new immunotherapeutic agent. *Expert Opin Investig Drugs*. 2010;19:141-149.
68. Stein R, Qu Z, Cardillo TM, et al. Antiproliferative activity of a humanized anti-CD74 monoclonal antibody, hLL1, on B-cell malignancies. *Blood*. 2004;104:3705-3711.
69. Stein R, Smith MR, Chen S, Zalath M, Goldenberg DM. Combining milatuzumab with bortezomib, doxorubicin, or dexamethasone improves responses in multiple myeloma cell lines. *Clin Cancer Res*. 2009;15:2808-2817.
70. Sapra P, Stein R, Pickett J, et al. Anti-CD74 antibody-doxorubicin conjugate, IMMU-110, in a human multiple myeloma xenograft and in monkeys. *Clin Cancer Res*. 2005;11:5257-5264.
71. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12:252-264.
72. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.
73. Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 2003;100:4712-4717.
74. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
75. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.
76. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793-800.
77. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
78. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
79. Vonderheide RH. Prospect of targeting the CD40 pathway for cancer therapy. *Clin Cancer Res*. 2007;13:1083-1088.
80. Law CL, Gordon KA, Collier J, et al. Preclinical antilymphoma activity of a humanized anti-CD40 monoclonal antibody, SGN-40. *Cancer Res*. 2005;65:8331-8338.
81. Advani R, Forero-Torres A, Furman RR, et al. Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma. *J Clin Oncol*. 2009;27:4371-4377.
82. Luqman M, Klabunde S, Lin K, et al. The antileukemia activity of a human anti-CD40 antagonist antibody, HCD122, on human chronic lymphocytic leukemia cells. *Blood*. 2008;112:711-720.
83. Byrd JC, Kipps TJ, Flinn IW, et al. Phase I study of the anti-CD40 humanized monoclonal antibody lucatumumab (HCD122) in relapsed chronic lymphocytic leukemia. *Leuk Lymphoma*. 2012;53:2136-2142.
84. Vonderheide RH, Flaherty KT, Khalil M, et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol*. 2007;25:876-883.
85. Beatty GL, Chiorean EG, Fishman MP, et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. 2011;331:1612-1616.
86. Lee JC, Chow NH, Wang ST, Huang SM. Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. *Eur J Cancer*. 2000;36:748-753.
87. Ohm JE, Gabrilovich DI, Sempowski GD, et al. VEGF inhibits T cell development and may contribute to tumor-induced immune suppression. *Blood*. 2003;101:4878-4886.
88. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med*. 2006;355:2542-2550.
89. Kreisl TN, Kim L, Moore K, et al. Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *J Clin Oncol*. 2009;27:740-745.
90. Vredenburgh JJ, Desjardins A, Herndon 2nd JE, et al. Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. *J Clin Oncol*. 2007;25:4722-4729.

91. Miller K, Wang M, Gralow J, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med*. 2007;357:2666-2676.
92. Robert NJ, Dieras V, Glaspy J, et al. RIBBON-1: randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2-negative, locally recurrent or metastatic breast cancer. *J Clin Oncol*. 2011;29:1252-1260.
93. Brufsky AM, Hurvitz S, Perez E, et al. RIBBON-2: a randomized, double-blind, placebo-controlled, phase III trial evaluating the efficacy and safety of bevacizumab in combination with chemotherapy for second-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. *J Clin Oncol*. 2011;29:4286-4293.
94. Pivot X, Schneeweiss A, Verma S, et al. Efficacy and safety of bevacizumab in combination with docetaxel for the first-line treatment of elderly patients with locally recurrent or metastatic breast cancer: results from AVADO. *Eur J Cancer*. 2011;47:2387-2395.
95. Spratlin J. Ramucirumab (IMC-1121B): Monoclonal antibody inhibition of vascular endothelial growth factor receptor-2. *Curr Oncol Rep*. 2011;13:97-102.
96. Huang Y, Chen X, Dikov MM, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood*. 2007;110:624-631.
97. Spratlin JL, Cohen RB, Eadens M, et al. Phase I pharmacologic and biologic study of ramucirumab (IMC-1121B), a fully human immunoglobulin G1 monoclonal antibody targeting the vascular endothelial growth factor receptor-2. *J Clin Oncol*. 2010;28:780-787.
98. Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer*. 2010;10:505-514.
99. Xing F, Saidou J, Watabe K. Cancer Associated Fibroblasts (CAFs) in tumor microenvironment. *Front Biosci*. 2010;15:166-179.
100. Kraman M, Bambrough PJ, Arnold JN, et al. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science*. 2010;330:827-830.
101. Hofheinz RD, al-Batran SE, Hartmann F, et al. Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibrotuzumab in patients with metastatic colorectal cancer. *Onkologie*. 2003;26:44-48.
102. Santos AM, Jung J, Aziz N, Kissil JL, Pure E. Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *J Clin Invest*. 2009;119:3613-3625.
103. Ostermann E, Garin-Chesa P, Heider KH, et al. Effective immunoconjugate therapy in cancer models targeting a serine protease of tumor fibroblasts. *Clin Cancer Res*. 2008;14:4584-4592.
104. Topp MS, Kufer P, Gokbuget N, et al. Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival. *J Clin Oncol*. 2011;29:2493-2498.
105. Seimetz D, Lindhofer H, Bokemeyer C. Development and approval of the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3) as a targeted cancer immunotherapy. *Cancer Treat Rev*. 2010;36:458-467.
106. Ruf P, Lindhofer H. Induction of a long-lasting antitumor immunity by a trifunctional bispecific antibody. *Blood*. 2001;98:2526-2534.
107. Strohlein MA, Siegel R, Jager M, Lindhofer H, Jauch KW, Heiss MM. Induction of anti-tumor immunity by trifunctional antibodies in patients with peritoneal carcinomatosis. *J Exp Clin Cancer Res*. 2009;28:18.
108. Jager M, Schoberth A, Ruf P, et al. Immunomonitoring results of a phase II/III study of malignant ascites patients treated with the trifunctional antibody catumaxomab (anti-EpCAM x anti-CD3). *Cancer Res*. 2012;72:24-32.
109. Horton HM, Bernett MJ, Pong E, et al. Potent in vitro and in vivo activity of an Fc-engineered anti-CD19 monoclonal antibody against lymphoma and leukemia. *Cancer Res*. 2008;68:8049-8057.
110. Gong R, Wang Y, Feng Y, Zhao Q, Dimitrov DS. Shortened engineered human antibody CH2 domains: increased stability and binding to the human neonatal Fc receptor. *J Biol Chem*. 2011;286:27288-27293.
111. Niwa R, Sakurada M, Kobayashi Y, et al. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. *Clin Cancer Res*. 2005;11:2327-2336.
112. Mossner E, Brunker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood*. 2010;115:4393-4402.
113. Salles G, Morschhauser F, Lamy T, et al. Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. *Blood*. 2012;119:5126-5132.
114. Sehn LH, Assouline SE, Stewart DA, et al. A phase 1 study of obinutuzumab induction followed by 2 years of maintenance in patients with relapsed CD20-positive B-cell malignancies. *Blood*. 2012;119:5118-5125.

Patrick Hwu, Gregory Lizée, and Yong-Jun Liu

51

Cancer and the Cellular Immune Response

Introduction

Key features of the immune system are its abilities to distinguish self from nonself, to recognize and respond to a myriad of foreign molecules (antigens) with exquisite specificity, to remember previously encountered antigens and quickly mobilize an expanded response, and to scan the entire body continuously for antigens. These properties make the immune system ideal for defending the host against developing and recurrent cancers and nascent metastatic tumor deposits, at least in theory. In spite of its promise, however, the application of immunological approaches to the cancer problem has only recently started to yield the dramatic results long anticipated by immunologists.[1-4] The major obstacle to using the immune system and its various components to prevent or treat human cancer has been a lack of understanding of the fundamental mechanisms that govern the immune response. These include the mechanisms by which cells of the immune system recognize antigens, expand and differentiate, find and destroy aberrant cells and pathogens, and die or become quiescent when no longer needed. The inability to direct these processes toward the destruction of cancer cells in the body in a consistent and effective way has thwarted many attempts at cancer immunotherapy to date. However, there are now a number of clear instances in which immunological approaches have brought significant benefit to cancer patients. This progress sustains the belief that the immune system has much to offer in controlling cancer, if only we could better control and direct its destructive powers.

Very recently, major advances have been made in understanding how cells of the immune system become activated on encountering an antigen and how they communicate with their external environment and with each other. Some of these advances are a direct result of the Human Genome Project, which has enabled the discovery of previously unknown genes coding for families of cytokines and receptors involved in regulating inflammatory and immune responses.[5] Others are derived from insights gleaned from studies of how the immune system defends the host against microbial pathogens. The normal host has various mechanisms that help rid it of invading pathogens.[6] Collectively, these mechanisms are called *innate* or *natural immunity*, and they require no previous contact with the pathogen to become active. *Acquired immunity*, on the other hand, is an immune response triggered by an encounter with a foreign cell or molecule and involves an amplification process that increases the number of lymphocytes able to recognize and respond to an invader. Until recently, the means by which innate immune mechanisms distinguish pathogens from self were unknown. A number of studies have demonstrated that the innate immune system recognizes many lethal microbial pathogens by means of pattern recognition receptors (Toll-like receptors, TLRs) on antigen-presenting cells (APCs).[7-10] These receptors recognize common molecular patterns shared among bacteria or viruses but not present on normal or cancerous host cells, such as lipoprotein, peptidoglycan, lipopolysaccharide, lipoteichoic acid, bacterial DNA, and viral double-stranded RNA. The triggering of pattern recognition receptors by such molecules has three important effects on the APCs of the immune system: (1) expression of stable and high levels of pathogen-derived peptide-MHC complexes on the cell surface, which will trigger T-cell responses; (2) expression of high levels of co-stimulatory molecules such as CD80 and CD86 that prime and activate antigen-specific T cells; and (3) secretion of a large number of proinflammatory cytokines, such as interleukin 1 (IL-1), IL-6, IL-12, TNF-α, GM-CSF, and type 1 interferon.[7-10] The proinflammatory cytokines further activate APCs, directly activate the innate killer cells of the immune system (NK cells and macrophages), and promote T-cell differentiation into antigen-specific helper T cells or cytotoxic T cells that mediate acquired immunity. These new findings explain the mechanism of action of bacterial adjuvants, which have long been used in animal models to enhance immunization toward protein and peptide antigens. They also suggest that the relative inefficiency of immunization with tumor antigens may be due, in part, to failure of the vaccines to stimulate the innate arm of the immune system, which is required for maximal amplification of antigen-specific, T-cell–mediated (acquired) immune responses. Thus, developing approaches to trigger

innate immunity in concert with more traditional approaches to stimulate acquired immunity represents a promising new strategy for cancer immunotherapy.

Other important advances concern the biology and activities of dendritic cells (DCs).[11-13] These are a heterogeneous group of cells involved in antigen detection, capture, processing, and presentation to T cells. DCs are the major cell type expressing germline-encoded immunoreceptors (TLRs, C-type lectin-like receptors, Ig-like molecules), and they are at the forefront of host defense mechanisms against microbial pathogens and altered self-molecules, such as those as encountered in virus-infected cells and autoimmune and antitumor responses.

Innate Recognition of Microbial Pathogens by Toll-Like Receptors

Recognition of Pathogen-Associated Molecular Patterns

An essential role of our immune system is to sense and protect us from infection by pathogenic microorganisms and establish tolerance to self-antigens. One of the most important mechanisms that the immune system distinguishes "infectious non-self" from "self" is the development of recognition of *pathogen-associated molecular patterns* (PAMPs).[6] This PAMPs recognition system was first identified in *Drosophila* as Toll, an essential molecule for embryonic patterning, as well as for antifungal immunity.[14] The first mammalian homologue of Toll was identified as Toll-like receptor 4 (TLR-4), which was shown to recognize lipopolysaccharides (LPS) from gram-negative bacteria. Signaling TLR-4 triggers APCs to upregulate MHC class I/class II molecules and co-stimulatory molecules, as well as secrete proinflammatory cytokines, and therefore enable DCs to activate T-cell–mediated adaptive immune responses.[15,16] To date, 10 TLR family members have been found in humans and 12 TLR family members in mice, which recognize the pathogen-associated molecular patterns, including proteins, lipids, and nucleic acids.

TLR Structure

TLRs are type 1 transmembrane proteins. The extracellular portion of TLRs contains the leucine-rich repeats, which are essential for ligand binding. The intracellular portion shares a common structure with IL-1 receptor, called the Toll/IL-1 receptor homologous (TIR) domain, which is critical for signal transduction. The TLR genes are dispersed throughout the genome, with TLR1 and TLR6 genes on chromosome 4p14, TLR2 and TLR3 on 4q3.3-q35, TLR4 on 9q32-q33, TLR5 on 1q33.3-q42, TLR7 and TLR8 on Xq22, and TLR9 on 3q21.3.[7-9]

TLR Subdivision and Ligands

Phylogenetic analyses of the amino acid sequence and structure of TLRs, in combination with analyses of TLR ligand binding, suggest that TLRs evolved along three major pathways (Figure 51-1):

1. Recognition of nucleic acid: TLR7 and TLR8 recognize single-stranded viral RNA; TLR9 recognizes

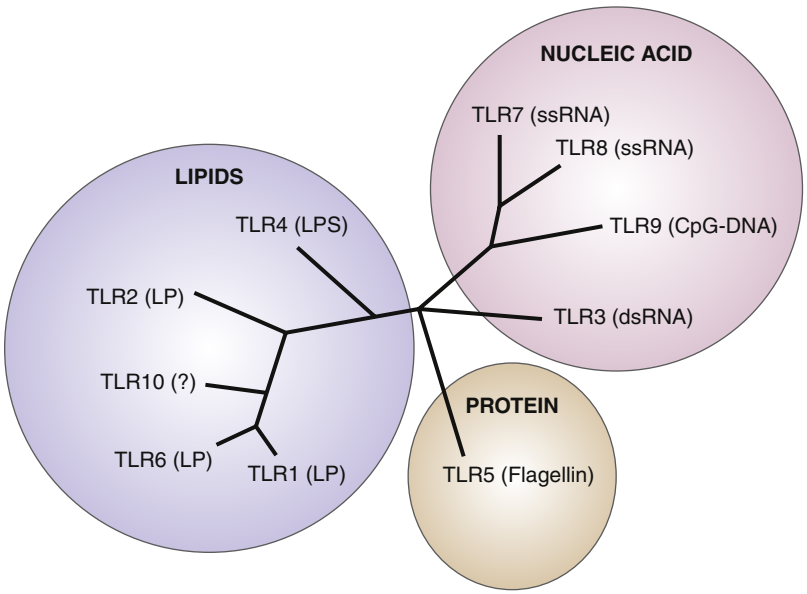


Figure 51-1 Toll-like receptor (TLR) family subdivision Phylogenetic analyses of the amino acid sequence and structure of TLRs suggest that they are evolved to recognize a variety of pathogen-derived signals such as microbial nucleic acids, lipids, and proteins.

double-stranded bacterial and viral DNA. TLR3 recognizes double-stranded viral RNA.

2. Recognition of lipid and lipoprotein: TLR4 recognizes LPS; TLR1, TLR2, and TLR6 recognize bacterial lipoprotein.
3. Recognition of protein: TLR5 recognizes bacterial flagellin.

Dendritic Cells Link Innate and Adaptive Immunity

Dendritic cells are specialized antigen-presenting cells, which display an extraordinary capacity to stimulate naïve T cells and initiate primary immune responses.[11,12] This established function of DCs has now offered the hope to apply DC-based immunotherapy for cancers. Recent studies suggest that DCs also play critical roles in the induction of peripheral immunological tolerance, regulate the types of T-cell immune responses, and function as effector cells in innate immunity against microbes. In both humans and mice, DCs can be grouped into two major subsets: the myeloid conventional DCs (mDC) and the plasmacytoid DCs (pDCs). Both display functional plasticity depending on the types of activation signals and also their resident microenvironments. However, mDCs and pDCs display different sets of TLRs and appear to regulate innate and adaptive immunity in different ways (Figure 51-2).

Myeloid DCs and Plasmacytoid DCs Express Different Sets of TLRs

In humans, mDCs can be identified by CD4+CD11c+lineage and pDCs can be identified by CD4+CD11c-lineage-BDCA-2+ILT7+. Strikingly, whereas mDCs express TLR2, 3, 4, 5, 6 and 8, pDCs express only TLR7 and TLR9. In response to the microbial ligands for TLR2, 3, 4, 5, 6 and 8, mDCs produce the TH1 polarizing cytokine IL-12 and cytokines IL-1, IL-6, IL-10, and TNF-α and undergo maturation by upregulation of MHC class I/class II and co-stimulatory cytokines CD80, CD83, and CD86.[10-12] In response to microbial ligands for the TLR7 and TLR9 and viral infection, pDCs rapidly produce massive amounts of type 1 IFNs, including IFN-α, IFN-β, and IFN-ω. Therefore, pDCs are also known as specialized type 1 IFN-producing cells (IPC), which represent the key cell type in antiviral innate immunity. pDCs also have the ability to produce IL-6 and TNF-α on activation through TLR7 and TLR9 and undergo differentiation into mature DCs that express high MHC class I/class II and co-stimulatory molecules CD80/CD86 and acquire the ability to prime naïve T-cell activation.[10-12]

Functional Plasticity of DCs

In humans, DCs were found to display different effector functions in directing T-cell responses that are regulated by

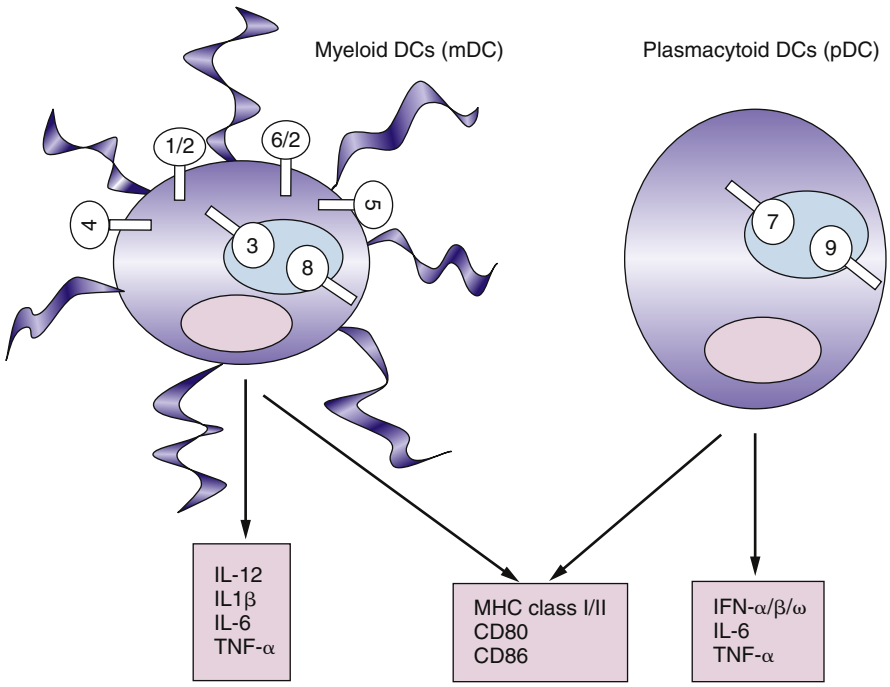


Figure 51-2 Toll-like receptor (TLR) expression in human dendritic cell subsets Myeloid DCs (mDC) and plasmacytoid DCs (pDC) express different sets of TLRs. Whereas mDCs preferentially express TLRs 1, 2, 3, 4, 5, 6, and 8, pDCs preferentially express TLRs 7 and 9. TLR stimulation of both DC subsets leads to the upregulation of MHC class I and class II molecules and T-cell co-stimulatory molecules CD80 and CD86. However, the two DC subsets express distinct cytokine profiles in response to TLR recognition, with mDCs producing IL-12 and pDCs producing primarily type I interferons.

the maturation stage of DCs and the maturation signals.[11,12] Whereas mDCs at the mature stage induce T_H1 differentiation and strong cytotoxic T lymphocyte (CTL) responses, mDCs at the immature stage induce IL-10–producing $CD4^+$ and $CD8^+$ regulatory T cells. Two groups of signals were shown to stimulate immature mDCs to induce T_H1 differentiation: (1) LPS derived from gram-negative bacteria (TLR4-L), gram-positive bacteria *Staphylococcus aureus* (SAC) (may trigger multiple TLRs), and double-stranded viral RNA (TLR3-L); and (2) T-cell signals such as CD40L and IFN-γ. Several signals were shown to stimulate immature mDC to induce T_H2 differentiation, including epithelial cell–derived cytokine TSLP and helminth *Schistosoma mansoni* egg antigen.[17,18] pDC-derived mature DCs also display different effector functions depending on the types of differentiation factors. Whereas pDCs activated by IL-3 and CD40L preferentially promote T_H2 differentiation, pDCs activated through TLR7 or TLR9 prime naïve T cells to produce IFN-γ and IL-10.[12]

Targeting TLRs on DCs to Induce Effective Antitumor Immunity

A major class of adjuvants for vaccines used in humans or in experimental animal models are killed microbials or microbial-derived products that trigger different TLRs.[19] The current understanding of TLR biology and DC biology reveals that the immune system has been evolved to fight against microbial pathogens, but does not have an optimal system for sensing and effectively responding to cancer. Therefore, a basic principle of developing a cancer vaccine is to instruct DCs to recognize tumor antigens as foreign by introducing microbial-derived adjuvants together with tumor antigens (Figure 51-3). In animal models, stimulation of TLR9 mainly expressed on plasmacytoid DCs with CpGs has been shown to increase the immunogenicity of different forms of cancer vaccines, including peptide vaccines, DNA vaccines, tumor cell–based vaccines, and DC-based vaccines.[19] Stimulation of TLR4 (mainly expressed

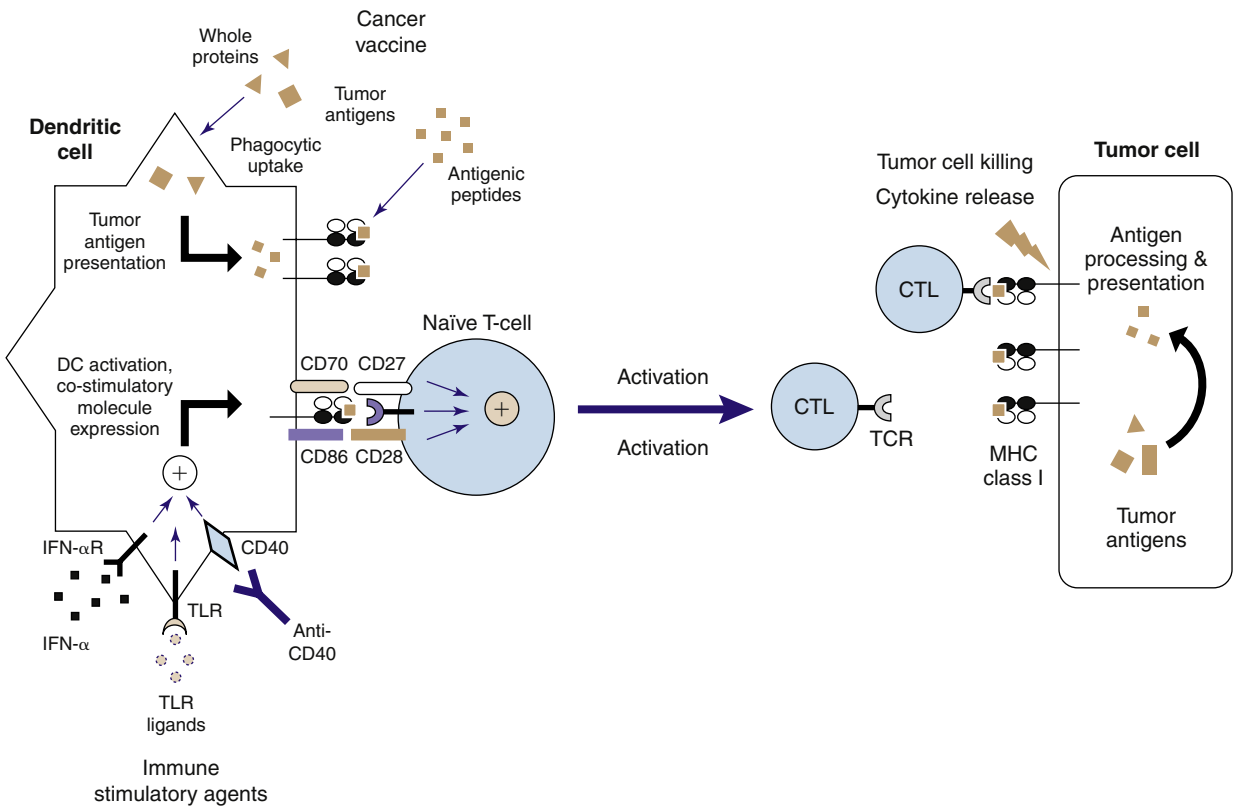


Figure 51-3 Generation of antitumor immune responses through dendritic cell–mediated T-cell priming Tumor-associated peptide antigens (TAA, *red*) expressed in the context of surface MHC class I (HLA) molecules on the surface of tumor cells can activate TAA-specific T cells to perform cytolytic functions and/or release inflammatory cytokines to further amplify the adaptive immune response. Generation of such tumor-reactive T cells requires stimulation of naïve T cells by activated dendritic cells (DCs) expressing both the appropriate TAA and co-stimulatory molecules such as CD86 and CD70. These signals together lead to the activation, proliferation, and trafficking of TAA-specific T cells to the tumor site, where they can induce tumor regressions. Cancer vaccines can introduce TAAs into DCs in several ways, including nucleic acids coding for TAAs, tumor cell lysates, whole TAA proteins, or TAA-derived peptides. DCs can then process and present these TAAs to naïve T cells in the context of MHC class I molecules. However, optimal T-cell activation requires the expression by DCs of co-stimulatory ligands for CD27 and CD28, which are known to be upregulated by the combination of TLR stimulation or interferon-α, along with ligation of the CD40 receptor. Thus, the generation of an optimal antitumor T-cell response requires a combination of TAA-specific vaccination with DC activation signals provided by TLR ligands and CD40-specific antibody.

by mDCs) by MPL, BCG, or murine β-defensin can also promote the immunogenicity of cancer vaccines. Several studies suggest that the ability of mDCs to present antigens and activate antigen-specific T cells can be greatly enhanced by activated pDCs through a type-1 IFN-dependent mechanism in both antiviral immune responses and autoimmune responses.[20] We have shown that pDCs activated by CpG promote the ability of mDCs to present melanoma antigens to T cells and induce strong tumor-specific CTL responses in vitro and in vivo. In addition, pDCs activated by CpG also strongly activate NK cells, which kill tumor cells and further enhance the ability of mDCs to take up dead tumor cells and cross-present tumor antigens to $CD8^+$ T cells.[21]

The Nature of Antitumor Immunity

T Cells Can Recognize Self-Antigens Expressed by Tumors

Over the past 10 years, numerous tumor antigens have been described that can be recognized by T cells.[22] These have been identified by two major methods: (1) molecular cloning using tumor antigen–specific T cells derived from cancer patients, and (2) analysis of candidate antigens based on gene expression and molecular profiling of tumors. Many of these antigens are expressed on normal tissues and are therefore considered to be "self" antigens. Examples of this class of antigen include melanocyte differentiation antigens that are expressed on melanoma cells as well as normal melanocytes. These include tyrosinase, MART-1, gp100, and TRP-1.[22] Differentiation antigens, expressed on tumor as well as the normal tissue of origin, can be targeted in immunotherapeutic strategies, as long as the normal tissue is nonessential. Mutated antigens, endogenous retroviral antigens, and antigens expressed in tumor and testis have also been described to be expressed in the context of MHC class I and class II molecules, capable of being recognized by $CD8^+$ and $CD4^+$ T cells, respectively. Although mutated antigens may be more easily recognized than "self" antigens, it is also possible that peripheral tolerance develops against the mutations, because co-stimulation may be absent in tumors, which are often present for many years before clinical diagnosis.

Autoimmune Vitiligo and Response to Immunotherapy

Because many tumor antigens are also expressed by normal host tissues, such as normal melanocytes in the case of melanoma antigens, obtaining an effective antitumor immune response requires overcoming self-tolerance. Indeed, in some settings, effective immunotherapy has been correlated with autoimmune responses against host tissues. IL-2, a cytokine that can stimulate the proliferation of T cells, can result in significant long-lasting regression of disease in some patients with metastatic melanoma and renal cell cancer (see later discussion). Interestingly, Rosenberg found that whereas 0 of 104 renal cancer patients treated with high-dose IL-2 developed vitiligo, the immune destruction of normal melanocytes, 11 of 74 melanoma patients treated with high-dose IL-2 developed vitiligo.[23] Furthermore, vitiligo was seen in 26% of melanoma patients who demonstrated objective tumor response to IL-2, whereas no vitiligo was observed in patients who did not respond to the IL-2. This suggests that tolerance to self-antigens can be overcome in the induction of an effective antitumor immune response.

Cancer Vaccines, Cytokines, and Immunotherapy

Cytokine Therapy of Cancer

Perhaps the strongest evidence that immune responses can result in a significant antitumor effect in patients is the fact that a subset of patients with metastatic melanoma can have complete tumor regressions and long-term survival following the administration of the T-cell growth factor IL-2. Of 270 patients with metastatic melanoma treated with high-dose IL-2, 43 (16%) had an objective response (complete or partial response).[24] More importantly, 60% of those patients who achieved a complete response had prolonged disease-free intervals and long-term survival (more than 10 years). This demonstrates that it is possible, by activation of the immune system, to induce clinically meaningful responses in patients. Future studies are needed to focus on understanding more fully the mechanism of response in patients so that improved strategies can be designed that will result in higher response rates and improved survival.

Another cytokine with significant clinical activity is interferon alpha, a type I interferon that is normally produced by plasmacytoid dendritic cells following viral infections. In randomized trials, high-dose interferon therapy has been shown to decrease tumor recurrence and increase survival in stage III melanoma patients (following surgical resection of tumor-positive lymph nodes).[25] Another study investigated prognostic markers in melanoma patients receiving interferon alpha in the adjuvant setting. Patients who developed autoantibodies during interferon therapy, including antithyroid, antinuclear, or anticardiolipin antibodies, had significantly enhanced survival compared to patients who did not develop signs of autoimmunity.[26] This again highlights the link between immunotherapy and autoimmunity as discussed earlier. In addition, it suggests that although interferon has

pleiotropic effects on tumor and host tissues, such as effects on tumor vasculature and direct inhibitory effects on tumor proliferation, the mechanism of action in melanoma patients is by stimulating antitumor immunity by breaking tolerance to self-antigens. This may be due to the effects of type I interferons on antigen-presenting cells or T cells. In addition, interferon alpha can upregulate MHC molecules on tumor cells, thereby rendering them better targets for T cells.

Current Status of Cancer Vaccines

With clear evidence that the immune system can play an important role in mediating clinical responses in cancer patients, current efforts are focused on developing cancer vaccines in order to enhance efficacy as well as specificity, because nonspecific immune stimulation can result in autoimmunity, as discussed. Two major approaches have been pursued in cancer vaccine strategies: the use of whole tumor cells or the use of specific tumor antigens (Table 51-1).

Whole-Cell Cancer Vaccine Strategies

Cancer vaccine strategies have been performed using derivatives of both autologous and allogeneic tumor cells. Although the use of autologous tumor cells is more labor intensive, in that vaccines need to be prepared individually for each patient, autologous tumor has the advantage of containing specific mutations for that patient, which may be seen as more foreign compared to shared self antigens. Cell lysates fed to autologous dendritic cells, isolation of heat shock proteins bound to autologous antigens, and gene modification of autologous tumor with immune-enhancing cytokines have been evaluated in clinical trials. In murine models, transduction of tumor cells to express GM-CSF results in enhanced antitumor immune responses against parental non-transduced tumor cells.[27] Antitumor activity of GM-CSF–expressing tumors was found to be dependent on host bone-marrow–derived antigen-presenting cells, CD1d-restricted NK T cells, $CD4^+$ and $CD8^+$ T cells, and antibodies.[28,29]

Antigen-Specific Vaccine Approaches

As discussed earlier, numerous tumor antigens have now been identified that can be recognized by T cells in the context of MHC class I and class II molecules. Many of these antigens are shared among specific types of tumors, and represent a feasible target for vaccine development. Antigen-specific vaccine approaches have included the use of specific peptide epitopes, whole proteins, and recombinant DNA and viral vaccines. The advantage of whole protein and recombinant approaches

Table 51-1 Advantages and Disadvantages of Various Cancer Vaccine Approaches

	Whole-Tumor Vaccines		Antigen-Specific Vaccines			
	Autologous	**Allogeneic**	**Peptide**	**Whole Protein**	**DNA**	**Recombinant Viral**
Advantages						
Easily produced		X	X		X	
Contains shared antigens, so can be used on multiple patients		X	X	X	X	X
Contains multiple antigens, including unknown antigens	X	X				
Contains multiple epitopes from the same antigen	X	X		X	X	X
Contains individual mutated antigens	X					
Disadvantages						
Costly	X			X		X
Needs to be produced for individual patients	X					
Stimulates a neutralizing antibody						X
Low levels of antigen expression	X	X			X	
Potentially stimulates competing antiviral immune responses						X

using the entire antigen gene is that multiple class I and class II epitopes may be presented.[30] However, whole proteins have been expensive and challenging to produce clinically, and viral vaccines can induce neutralizing antibodies that prevent the efficacy of serial doses of vaccine. Peptide vaccines, in combination with specific adjuvants, have demonstrated potential clinical efficacy in the case of chronic myelogenous leukemia and have been the most consistent method of inducing high levels of circulating antigen-specific T cells.[31,32] However, in the case of patients with metastatic melanoma, high levels of tumor-specific T cells in the blood do not always result in tumor regression.[33] Therefore, future efforts are focused on stimulating stronger and more effective T-cell priming through the use of specific adjuvants such as TLR agonists, using concepts learned from basic studies of antiviral immunity as described earlier. This may result in T cells with increased affinity and specificity as well as enhanced memory and effector function. One method to more carefully manipulate the specific phenotype of tumor-reactive immune cells is to generate and select specific T cells in the laboratory followed by reinfusion into patients. This is termed *adoptive immunotherapy*.

Adoptive Immunotherapy of Cancer

One of the most significant recent advances in clinic immunotherapy has been the adoptive transfer of tumor-reactive lymphocytes. A number of lines of evidence have demonstrated the clinical effectiveness of this approach, including donor-lymphocyte infusion following allogeneic bone marrow transplantation, treatment of metastatic EBV-driven lymphoproliferative tumors, and therapy of metastatic melanoma.[34,35]

Tumor-Infiltrating Lymphocytes

In the setting of metastatic melanoma, T cells can be found at the tumor site (tumor-infiltrating lymphocytes, or TILs) that are specific for melanoma antigens, such as MART-1 and gp100 (Figure 51-4). Because the tumor is growing, either these T cells are nonfunctional or the tumor is resistant to recognition or lysis. However, when TILs are expanded ex vivo and reinfused, clinical regressions are seen in patients with metastatic disease. A number of reasons may explain the effectiveness of ex vivo expanded lymphocytes compared to endogenous T cells. First, large numbers can be generated in the laboratory that may be difficult to achieve in vivo. Second, expanding the lymphocytes ex vivo takes them out of the suppressive tumor microenvironment. Finally, growth ex vivo may allow reactivation of lymphocytes rendered anergic or nonfunctional by in vivo toleragenic mechanisms. Initially, transfer of tumor-reactive T cells alone resulted in response rates of greater than 20%, but clinical regressions were often transient, and it was clear from gene marking studies that the T cells did not survive long in vivo.[36,37]

More recently it was found that transient lymphodepletion using cytoxan and fludarabine before T-cell infusion resulted in improved response rates and T-cell survival. Eighteen of 35 patients (51%) with metastatic melanoma exhibited objective responses following lymphodepletion and adoptive T-cell transfer.[38,39] Substantial numbers of infused lymphocytes were found in the circulation in some patients more than 2 years after infusion. This dramatic improvement following lymphodepletion may be due to a number of potential mechanisms including elimination of regulatory T cells and enhancement of lymphocyte homeostatic proliferation.

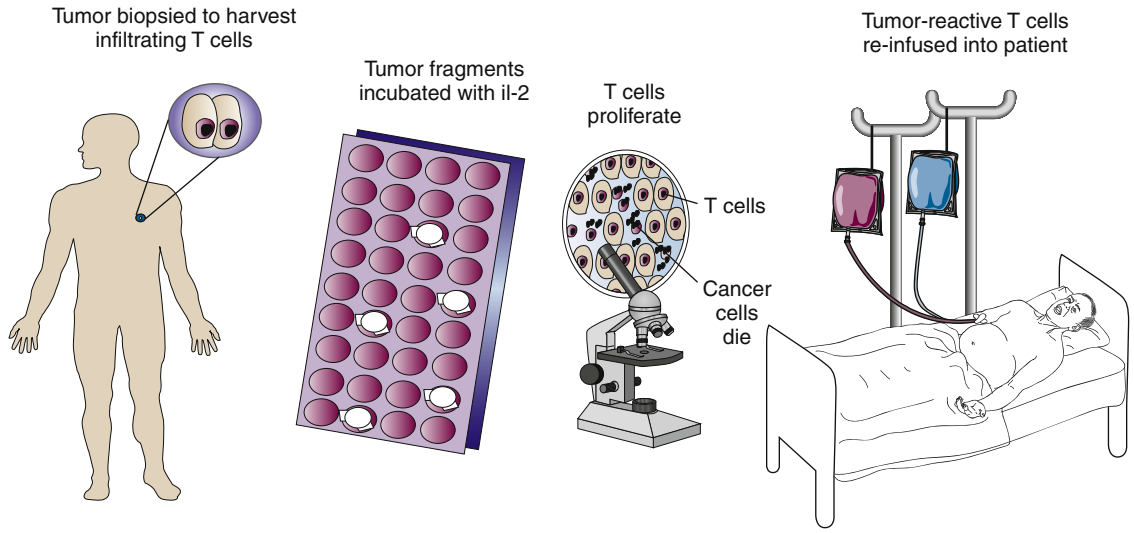


Figure 51-4 Expansion of tumor-infiltrating lymphocytes (TILs) from melanoma tumors T cells capable of specifically recognizing tumors can be found in some metastatic melanomas. When tumor fragments are cultured in the T-cell growth factor interleukin-2 (IL-2), the T cells expand and destroy the tumor cells in vitro. The expanded TIL can then be reinfused into metastatic melanoma patients in combination with interleukin-2.

Current studies of adoptive immunotherapy are focused on optimizing the generation of T cells ex vivo and their proliferation in vivo. For example, murine models suggest that proliferation of adoptively transferred T cells can be greatly enhanced in vivo by the addition of active immunization, such as dendritic cell vaccines.[40] In addition, significant efforts are focused on the introduction of novel genes into T cells in order to enhance their ability to recognize, migrate to, and eliminate tumor cells. In a recent study, nonspecific peripheral blood T cells were gene modified with a melanoma-specific T-cell receptor and then reinfused into melanoma patients. Two patients (of 17) demonstrated objective clinical responses, demonstrating that gene modification of lymphocytes is a feasible and potentially efficacious maneuver, although future studies are focused on enhancing the overall response rate.[41]

Gene-Modified T Lymphocytes

Because tumor-reactive lymphocytes are naturally found in melanomas, much of the work in adoptive therapy has focused on this disease. Although tumor-reactive lymphocytes are rarely found in other common cancers, antibodies that recognize these tumors in a relatively specific fashion have been described. Therefore, chimeric receptors have been designed using antibody variable regions extracellularly fused to T-cell signaling chains intracellularly (Figure 51-5). The initial studies demonstrated the ability to redirect T-cell specificity in vitro against ovarian cancer.[42] Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain, but subsequent receptors have been designed that recognize human immunodeficiency virus (HIV) as well as a number of other tumor types.[43-45]

Besides redirecting T-cell recognition, the introduction of novel genes into effector lymphocytes may be used to enhance other functional properties, such as T-cell migration to the site of tumor and activation status. For example, Kershaw and colleagues introduced the chemokine receptor gene CXCR2 into T cells and demonstrated the ability of these modified cells to migrate toward chemokines produced by tumor cells.[46] In addition, receptor genes have been introduced with signaling chains containing co-stimulatory sequences in order to enhance T-cell activation.[47]

In summary, the infusion of tumor-reactive, ex vivo expanded T cells has clearly demonstrated the effectiveness of T-cell–mediated immunity in the treatment of patients with metastatic cancer. Future studies will focus on enhancing response rates through generation of T cells with greater activity and ability to migrate to tumor, durability of response by improved maintenance of T cells in vivo, and the use of T cells in nonmelanoma tumors by redirecting cells with native T-cell receptor or novel chimeric receptor genes.

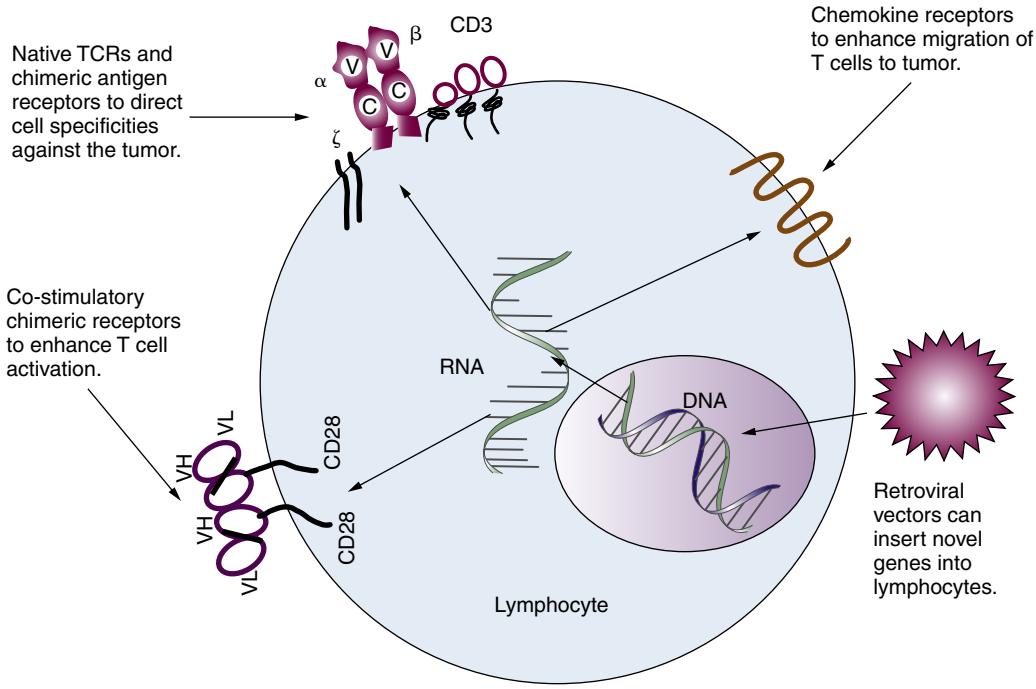


Figure 51-5 Insertion of genes into lymphocytes to enhance antitumor properties Genes can be inserted into T cells using retroviral vectors. These genes can endow the T cells with novel properties. Using genes encoding tumor antigen-specific T-cell receptors (TCRs) or chimeric antibody/T-cell receptor genes, T cells can gain the ability to recognize new targets. Using co-stimulatory receptor genes, T-cell activation can be further enhanced. Finally, introducing chemokine receptor genes into T cells can enhance T-cell migration to tumor sites.

Immune Regulatory Cells and Molecules in Human Cancer

Immunoregulatory cells and molecules are natural mechanisms that the immune system uses to prevent autoimmune destruction. Tumors often exploit these mechanisms to evade antitumor immunity, and several have been reported to be upregulated in human malignancy, including gastric cancer, ovarian cancer, melanoma, Hodgkin's disease, and kidney cancer (Table 51-2).

Regulatory T Lymphocytes in Human Cancer

$CD4^+D25^+$, naturally occurring regulatory T cells (Treg), constitute 5% to 10% of peripheral $CD4^+$ T cells, which play an essential role in the active suppression of autoimmunity in both humans and rodents. Treg appear to differentiate as a unique T-cell lineage in the thymus from immature T cells expressing T-cell receptor (TCR) with medium to high affinity for self-antigens, which depends on IL-2 and co-stimulatory molecules provided by activated APCs.[48-52] Foxp3, a member of the forkhead transcriptional factor family, has been demonstrated to be the master regulator of Treg development in the thymus, as well as Treg suppressive function. Increasing evidence suggests that tumor-specific Treg exist and play an essential role in immune tolerance to tumors and thus represent a major hurdle for antitumor immunotherapy. In addition, the tumor microenvironment appears to be the site where tumor-specific infiltrating T cells are actively converted into tumor-specific Treg.[53]

Table 51-2 Potentially Targetable Immunoregulatory Molecules

Molecule	Cellular Expression	Mechanism of Action
CTLA-4	Helper T Cytotoxic T	Provides co-inhibitory signaling during naive T-cell priming
	Treg	Induces local tryptophan metabolism by DCs, directly inhibits T cells
Membrane Bound		
PD-1	Helper T Cytotoxic T	Inhibits T-cell proliferation, cytokine production, and cytotoxicity
IL-10	Tumor Tri	Regulates growth and differentiation of a wide variety of immune cells
IL-13	iNKT	Induces immature myeloid cells to produce TGF-β
TGF-β	Tumor Tr1, Treg	Directly suppresses proliferation of antigen-activated T cells
	Immature myeloid	
Soluble		
VEGF	Tumor	Blocks DC differentiation and maturation, leading to accumulation of iDC and iMC
IDO	Tumor Dendritic	Depletes local tryptophan, inhibiting T-cell proliferation
ARG1	Tumor Immature myeloid	Depletes local arginine, inhibiting CD3f expression and T-cell activation
iNOS	Tumor Immature myeloid	Generates nitric oxide, inhibiting T-cell priming, proliferation, and cytotoxicity

Melanoma is perhaps the most immunogenic of solid tumors, as melanoma-specific $CD4^+$ and $CD8^+$ T cells can be isolated directly from tumor deposits. Yet the tumor metastases grow unabated, thereby implying that immune regulatory mechanisms may be preventing full activation and effector function by tumor-infiltrating T cells. Although it is not clear why melanoma-specific T cells found in tumor deposits are not functioning to eliminate the tumor in vivo, a number of studies have been performed in melanoma patients to help provide some insight into potential regulatory pathways that are active.

Indeed, $CD4^+CD25^+$ Treg have been isolated from melanoma deposits, and in fact some Treg lines have been found to be specific for LAGE-1 and ARCT1, expressed by melanoma cells.[54,55] In a DC melanoma vaccine study, Chakraborty and colleagues found that vaccine-induced specific CTL responses declined by day 28, and this was associated with expansion of $CD4^+CD25^+IL\text{-}10^+$ T cells.[56] $CD4^+CD25^+$ FoxP3 expressing cells were also found to be overrepresented in melanoma lymph-node metastases.[57] This may represent a mechanism by which tumors escape the immune system by first generating immunosuppression at the local lymph-node site.

Besides $CD4^+CD25^+$ Treg, melanoma cells themselves may produce factors or express receptors that can either induce regulatory immune cells or suppress effector T cells directly. For example, melanoma cells have been found to express IL-10, which is capable of inducing Tr-1 cells, another $CD4^+$ regulatory cell type that can induce T-cell anergy and suppression of immune responses, primarily via the production of high levels of IL-10 and TGF-β.[58,59]

A significant body of work has been performed in evaluating the cellular infiltrate of ovarian cancer. Zhang and co-workers performed immunohistochemical analysis of 186 advanced-stage ovarian cancer patients and found that the 5-year survival was 38% for patients whose tumors were infiltrated by $CD3^+$ T cells versus 4.5% for patients with

an absence of intratumoral T cells.[60] T-cell infiltration was associated with increased interferon-γ, interleukin-2, and specific chemokines within the tumor. However, another group found that not all $CD3^+$ T-cell subpopulations were favorably correlated with outcome in ovarian cancer patients. Accumulation of $CD4^+CD25^+$ Treg cells in ovarian cancer was found to predict decreased survival for both stage III and stage IV patients.[61] Wolf and associates confirmed and extended these findings by demonstrating that quantitative FoxP3 levels of ovarian biopsies by real-time PCR could identify a patient subgroup with decreased overall survival.[62] Finally, Sato and colleagues found that ovarian cancer patients with a higher $CD8^+$/Treg ratio in tumor tissue had an increased survival.[63] Together, these studies suggest that effector T cells play an important role in mediating an antitumor immune response in ovarian cancer patients, whereas Treg negatively influences this response. This implies that strategies to enhance tumor-specific $CD8^+$ T cells while decreasing Treg may be effective in improving the outcome for ovarian cancer patients.

Myeloid-Derived Suppressor Cells in Human Cancer

There is accumulating evidence that progressive tumor growth is correlated with an increased frequency of immature myeloid cells (iMC) and immature DCs (iDC) in the tumor microenvironment, which can inhibit the function of tumor-specific T lymphocytes.[64-66] These immature myeloid cells can be induced by tumor-derived factors such as vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor, IL-6, granulocyte-macrophage colony-stimulating factor, IL-10, and gangliosides. The proangiogenic cytokine VEGF is a major contributor to immune suppression, and tumors often produce large quantities of VEGF that can be detected in the serum of cancer patients.[67,68] In vitro studies have shown that VEGF can block DC maturation, leading to the production of iMCs; conversely, blocking VEGF promotes normal DC differentiation and enhances antitumor function.[69]

Tumors from patients with cancer often contain iDCs with reduced T-cell stimulatory capacity, often expressing low levels of the co-stimulatory molecules CD80 and CD86; further, they fail to upregulate them even in the presence of DC maturation factors.[70,71] Increased levels of circulating immature myeloid cells have also been observed in the peripheral blood of patients with lung, breast, head and neck, and esophageal cancer.[65,72] Importantly, patients with cancer often show a significant reduction in normal circulating levels of mature DCs that can be reversed on surgical removal of tumors.[73,74] Thus, abnormal differentiation and maturation of DCs in vivo, mediated by tumor-derived soluble factors, likely play a substantial role in preventing the effective priming of a productive, T-cell–mediated antitumor immune response. Circulating levels of iMCs have been well correlated with stage of disease and poorer prognosis, and surgical resection of tumors has been shown to decrease the number of peripheral blood iMCs in both human and animal models.[75-77]

Immature MCs mediate their immunosuppressive activity through the inhibition of IFN-γ production by $CD8^+$ T cells in response to MHC class I–associated peptide epitopes presented on the iMC surface.[78] This effect requires direct cell-to-cell contact and is mediated by reactive oxygen and nitrogen species, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO), secreted by the iMCs in close proximity to the T cell.[79,80] Although the precise mechanism of action on T cells has yet to be fully elucidated, there is some indication that iMCs act, in part, through downregulation of the CD3ζ chain on responding $CD8^+$ T cells.[81,82] A population of iMCs has been described in the peripheral blood of cancer patients having high arginase-1 activity capable of depleting local arginine levels and down-modulating CD3ζ levels on T cells. Depletion of this iMC subset in vitro restored CD3 expression and normal T-cell responses.[83,84]

In summary, there is evidence to suggest that the balance of immature and mature myeloid cells can have a significant effect on both naturally occurring and vaccine-induced antitumor T-cell responses. It is becoming increasingly apparent that effective cancer immunotherapy may require the correction of aberrant myeloid cell differentiation frequently observed in tumor-bearing hosts.

Immune Checkpoint Blockade

The development of a new class of effective cancer immunotherapy agents has recently become possible because of advances in our understanding of T-cell activation and regulation. T-cell activation is initiated by interaction of the TCR with MHC-bound peptide antigens on APCs (see Figure 51-3). However, effective priming of naïve T cells also requires a second, co-stimulatory signal mediated by the binding of CD28 on the T-cell surface to CD80 or CD86 (Figure 51-6). These two signals together allow T cells to proliferate, acquire antitumor effector functions, and eventually migrate to disease sites for tumor-cell killing.[85] T-cell activation is tightly regulated by a number of inhibitory signals in order to avoid prolonged immune responses that can potentially damage normal tissues. Inhibitory signals that are intrinsic to T cells are known as *immune checkpoint molecules*, with

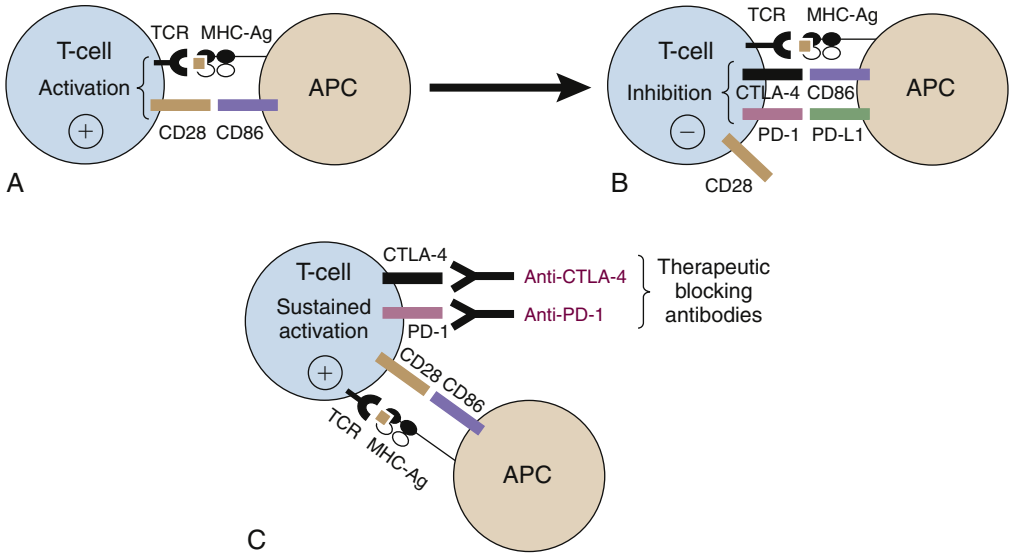


Figure 51-6 Immune checkpoint blockade therapies **(A)** T-cell activation is initiated by the interaction of the T-cell receptor (TCR) with major histocompatibility complex (MHC) molecules presenting antigen on an antigen-presenting cell (APC). Optimal activation of the T cell requires additional signals that are provided by the interaction between CD28 and CD86. **(B)** T-cell activation is naturally attenuated by upregulation of cytotoxic T lymphocyte–associated protein 4 (CTLA-4) on the surface of activated T cells, where it outcompetes CD28 for binding to CD86 on APCs. Additional regulation of T-cell activity is also provided by later inhibitory signals through programmed cell death 1 (PD-1), which binds to PD1 ligand 1 (PD-L1). **(C)** Strategies to sustain activated tumor-specific T cells include the use of blocking monoclonal antibodies targeting CTLA-4, PD-1, or PD-L1 to neutralize co-inhibitory receptors. Therapeutic antibodies that block intrinsic inhibitory immune checkpoints can allow for sustained T-cell effector responses, including increased production of cytokines and cytotoxic function.

the most well studied being cytotoxic T lymphocyte–associated protein 4 (CTLA-4) or programmed cell death 1 (PD-1).[85,86]

CTLA-4 Blockade

CTLA-4 is expressed by activated CD4 and CD8 T cells. It is a homologue of T-cell co-stimulator CD28 but has a higher binding affinity for its ligands. On T-cell activation, signaling pathways lead to the expression of CTLA-4, which is then mobilized from intracellular vesicles to the cell surface, where it outcompetes co-stimulator CD28 for binding to its ligands. Binding of CTLA-4 to CD86 proteins interrupts CD28 co-stimulatory signals and, as a result, limits T-cell responses (see Figure 51-6). In addition to the intrinsic restriction of effector T-cell responses due to expression of CTLA-4 on effector cells, there can also be extrinsic restriction of effector T-cell responses due to the expression of CTLA-4 on regulatory T cells as well.[87-89]

Because of the negative regulatory effects of CTLA-4 on T-cell responses, it was hypothesized that blockade of CTLA-4 signaling would potentiate antitumor immune responses. Indeed, anti-CTLA-4 antibodies were able to cause rejection of syngeneic transplanted tumors in mice.[90] These preclinical studies led to the development of an antibody to block human CTLA-4 (ipilimumab), which was shown in Phase III clinical trials to improve overall survival of patients with advanced melanoma.[91] Initial Phase I and Phase II clinical trials in other cancer patients demonstrated that ipilimumab treatment could also induce significant antitumor activity.[92,93] Subsequently, a Phase III randomized trial showed that ipilimumab improved the median overall survival of advanced metastatic melanoma patients by 3.7 months (10.1 vs. 6.4 months; $P = .003$).[91] However, the most striking feature of this study was the fact that nearly a quarter of the patients survived longer than 4 years. This trial led to the approval of ipilimumab by the U.S. Food and Drug Administration (FDA) in March 2011 for the treatment of patients with metastatic melanoma. Most recently, a second randomized, Phase III clinical trial showed that the addition of ipilimumab to standard dacarbazine chemotherapy significantly improved overall survival by 2.1 months in melanoma patients.[94] More importantly, anti-CTLA-4 therapy has demonstrated the long-awaited promise of immunotherapeutic agents to elicit durable responses in patients with many different types of cancers.

PD-1/PD-L1 Blockade

Another important T-cell checkpoint that can be targeted clinically is the interaction of PD-1 and its ligands (see Figure 51-6). PD-1 is mainly expressed by activated CD4 and CD8 T cells, and it has two ligands, PD-L1 and PD-L2, with distinct expression profiles.[95] PD-L1 is expressed not only on APCs, but also on T cells, B cells, and nonhematopoietic

cells, including tumor cells. Expression of PD-L2 is largely restricted to APCs, including macrophages and myeloid DCs. The role of PD-1 as a negative regulator of T and B cells was best demonstrated by the findings that PD-1–deficient mice developed significant autoimmunity.[96,97] Subsequently, blocking antibodies against PD-1 were shown to induce a reduction of tumor growth and metastasis in a number of experimental mouse models.[98,99] Consistent with the immune inhibitory role of PD-1/PD-L1/2 signaling, enforced expression of PD-L1 on tumor cells caused enhanced tumor growth in vivo, which could otherwise be kept in check by T cells. Again, this augmentation of tumor growth could be reversed with the use of blocking antibodies against PD-L1.[100]

Consistent with these preclinical studies, a recent Phase I clinical trial using an anti-PD-1 antibody (BMS-936558) showed an 18% to 28% objective response rate in patients with advanced non–small-cell lung cancer (NSCLC), renal call carcinoma (RCC), and melanoma.[101] In addition, another concurrent Phase I trial with anti-PD-L1 antibody demonstrated an objective response rate of 6% to 17% in patients with advanced NSCLC, melanoma, and RCC.[102] These early-phase clinical studies show a very promising potential for employing blocking antibodies against the inhibitory PD-1/PDL-1/2 signaling pathway in anticancer immunotherapy.

Checkpoint Blockade and the Induction of Autoimmunity

As with most experimental cancer therapies, treatment with immune checkpoint blockade agents is sometimes associated with a distinct set of on-target toxicities due to immune-related adverse events. For example, among melanoma patients treated with ipilimumab, up to 60% of patients manifested autoimmune reactions including dermatitis, colitis, hepatitis, and hypopituitarism.[91] Early recognition, diagnosis, and treatment of such drug-induced inflammatory conditions with steroids are critical for minimizing the adverse effects while maximizing the therapeutic benefits of this promising anticancer agent. By contrast, the early Phase I results targeting the PD-1 pathway have shown less overall toxicity in cancer patients, although future studies are required to confirm this. Regardless, investigations into biomarkers/pathways that are associated with or predict clinical benefit or toxicities will be important as the field of cancer immunotherapy moves forward, and recent studies in these areas have been promising.[103,104] Perhaps future strategies combining checkpoint blockade strategies with an effective cancer vaccine may skew the immune response toward tumor-specific antigens and away from normal tissue-associated self-antigens.

In summary, the recent clinical successes with immune checkpoint blockade agents have provided clear data to indicate that the immune system can be harnessed successfully to treat cancer and that the exquisite ability of the immune response to target tumor-specific antigens, as well as generate memory cells to thwart recurrences, can lead to durable clinical benefit in patients. These exciting study results and clinical benefits with anti-CTLA-4 and anti-PD-1 have provided a strong foundation on which to build for even greater success as we determine how to best select patients for treatment and how to combine immunotherapy with other agents to increase the number of patients who benefit.

Future Cancer Immunotherapies Will Use Basic Principles of Cellular Immunity

Although the adoptive transfer of carefully selected T-cell populations or the use of optimized vaccine strategies may allow for the presence of more potent T cells in vivo, this must be coupled with enhanced conditioning of the immunosuppressive tumor microenvironment to allow for both migration and function of specific T cells. In successful immune responses against viruses, a proinflammatory state exists in the infected site by TLR activation through specific viral components, as discussed earlier. This inflammation induces the upregulation of specific adhesion molecules on endothelial cells that will in turn mediate trafficking of primed T cells into the infected site. In addition, the proinflammatory state may enhance effector T-cell function while limiting the effects of immune regulation. Successful immunotherapy will require a better understanding of the interplay between immune-cell subpopulations that results in optimal T-cell induction as well as tumor site conditioning that will allow for enhanced efficacy of the T cells within the tumor microenvironment. Thus, the most effective strategies will likely involve combination approaches that aim to boost antitumor T-cell responses while simultaneously blocking immune inhibitory pathways. A number of immunosuppressive mechanisms are now targetable, including immune checkpoint blockade, depletion of regulatory immune cell subsets, and inhibition of oncogenic signaling.[105] This multidimensional approach will require exploring combinations of new and existing clinical agents to determine which are the most effective.

Finally, although many of the initial principles have been developed using more immunogenic tumors such as melanoma, future goals include the application of these same principles to develop successful immunotherapeutic approaches against other common cancers such as breast, lung, colon, and prostate cancer.

Key References

4. Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy. *Sci Transl Med*. 2012;4:127ps8.
17. Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med*. 2006;203:269-273.
19. van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. *Trends Immunol*. 2006;27:49-55.
20. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol*. 2005;23:275-306.
21. Liu C, Lou Y, Lizee G, et al. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. *J Clin Invest*. 2008;118:1165-1175.
25. Agarwala SS, Kirkwood JM. Update on adjuvant interferon therapy for high-risk melanoma. *Oncology (Williston Park)*. 2002;16:1177-1187; discussion 90-92, 97.
26. Gogas H, Ioannovich J, Dafni U, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. *N Engl J Med*. 2006;354:709-718.
29. Dranoff G. GM-CSF-secreting melanoma vaccines. *Oncogene*. 2003;22:3188-3192.
31. Molldrem JJ. Vaccination for leukemia. *Biol Blood Marrow Transplant*. 2006;12:13-18.
33. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909-915.
37. Rosenberg SA. Shedding light on immunotherapy for cancer. *N Engl J Med*. 2004;350:1461-1463.
39. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol*. 2005;23:2346-2357.
40. Lou Y, Wang G, Lizee G, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. *Cancer Res*. 2004;64:6783-6790.
41. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314:126-129.
48. Sakaguchi S. Naturally arising CD4$^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531-562.
50. Rudensky AY, Campbell DJ. In vivo sites and cellular mechanisms of T reg cell-mediated suppression. *J Exp Med*. 2006;203:489-492.
51. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4$^+$CD25$^+$ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.
53. Wang RF, Peng G, Wang HY. Regulatory T cells and Toll-like receptors in tumor immunity. *Semin Immunol*. 2006;18:136-142.
54. Wang HY, Lee DA, Peng G, et al. Tumor-specific human CD4$^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity*. 2004;20:107-118.
55. Wang HY, Peng G, Guo Z, et al. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4$^+$ regulatory T cells. *J Immunol*. 2005;174:2661-2670.
56. Chakraborty NG, Chattopadhyay S, Mehrotra S, et al. Regulatory T-cell response and tumor vaccine-induced cytotoxic T lymphocytes in human melanoma. *Hum Immunol*. 2004;65:794-802.
57. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4$^+$CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol*. 2004;173:1444-1453.
60. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med*. 2003;348:203-213.
61. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.
62. Wolf D, Wolf AM, Rumpold H, et al. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res*. 2005;11:8326-8331.
63. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8$^+$ tumor-infiltrating lymphocytes and a high CD8$^+$/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A*. 2005;102:18538-18543.
65. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol*. 2004;4:941-952.
66. Peranzoni E, Zilio S, Marigo I, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol*. 2010;22:238-244.
68. Laxmanan S, Robertson SW, Wang E, et al. Vascular endothelial growth factor impairs the functional ability of dendritic cells through Id pathways. *Biochem Biophys Res Commun*. 2005;334:193-198.
74. Della Bella S, Gennaro M, Vaccari M, et al. Altered maturation of peripheral blood dendritic cells in patients with breast cancer. *Br J Cancer*. 2003;89:1463-1472.
77. Danna EA, Sinha P, Gilbert M, et al. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. *Cancer Res*. 2004;64:2205-2211.
80. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8$^+$ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol*. 2004;172:989-999.
83. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res*. 2004;64:5839-5849.
84. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65:3044-3048.
85. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat Rev Cancer*. 2011;11:805-812.
86. Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. *Clin Exp Immunol*. 2009;157:9-19.
87. Ise W, Kohyama M, Nutsch KM, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol*. 2010;11:129-135.
88. Wing K, Yamaguchi T, Sakaguchi S. Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. *Trends Immunol*. 2011;32:428-433.
89. Corse E, Allison JP. Cutting edge: CTLA-4 on effector T cells inhibits in trans. *J Immunol*. 2012;189:1123-1127.
91. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
92. Callahan MK, Wolchok JD, Allison JP. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol*. 2010;37:473-484.
93. Wolchok JD, Neyns B, Linette G, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol*. 2010;11:155-164.
94. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011;364:2517-2526.
95. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
98. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol*. 2005;17:133-144.
99. Nomi T, Sho M, Akahori T, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res*. 2007;13:2151-2157.
101. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
102. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
103. Liakou CI, Kamat A, Tang DN, et al. CTLA-4 blockade increases IFNgamma-producing CD4$^+$ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A*. 2008;105:14987-14992.
104. Carthon BC, Wolchok JD, Yuan J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. *Clin Cancer Res*. 2010;16:2861-2871.
105. Khalili JS, Liu S, Rodriguez-Cruz TG, et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. *Clin Cancer Res*. 2012;18:5329-5340.

This page intentionally left blank

References

1. Pardoll DM. Cancer vaccines. *Immunol Today*. 1993;14:310-316.
2. Rosenberg SA. Progress in the development of immunotherapy for the treatment of patients with cancer. *J Intern Med*. 2001;250:462-475.
3. Greenberg PD. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol*. 1991;49:281-355.
4. Rosenberg SA. Raising the bar: the curative potential of human cancer immunotherapy. *Sci Transl Med*. 2012;4:127ps8.
5. Fahrer AM, Bazan JF, Papathanasiou P, et al. A genomic view of immunology. *Nature*. 2001;409:836-838.
6. Janeway Jr CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216.
7. Akira S. Toll-like receptors and innate immunity. *Adv Immunol*. 2001;78:1-56.
8. Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol*. 2001;1:135-145.
9. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature*. 2000;406:782-787.
10. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med*. 2001;194:863-869.
11. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.
12. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell*. 2001;106:259-262.
13. Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. *Int J Cancer*. 2001;94:459-173.
14. Lemaitre B, Nicolas E, Michaut L, et al. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*. 1996;86:973-983.
15. Medzhitov R, Janeway Jr CA. Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol*. 1997;9:4-9.
16. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*. 1998;282:2085-2088.
17. Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med*. 2006;203:269-273.
18. de Jong EC, Vieira PL, Kalinski P, et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. *J Immunol*. 2002;168:1704-1709.
19. van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. *Trends Immunol*. 2006;27:49-55.
20. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol*. 2005;23:275-306.
21. Liu C, Lou Y, Lizee G, et al. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. *J Clin Invest*. 2008;118:1165-1175.
22. Wang RF, Rosenberg SA. Human tumor antigens for cancer vaccine development. *Immunol Rev*. 1999;170:85-100.
23. Rosenberg SA, White DE. Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. *J Immunother Emphasis Tumor Immunol*. 1996;19:81-84.
24. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol*. 1999;17:2105-2116.
25. Agarwala SS, Kirkwood JM. Update on adjuvant interferon therapy for high-risk melanoma. *Oncology (Williston Park)*. 2002;16:1177-1187; discussion 90-92, 97.
26. Gogas H, Ioannovich J, Dafni U, et al. Prognostic significance of autoimmunity during treatment of melanoma with interferon. *N Engl J Med*. 2006;354:709-718.
27. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A*. 1993;90:3539-3543.
28. Huang AY, Golumbek P, Ahmadzadeh M, et al. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science*. 1994;264:961-965.
29. Dranoff G. GM-CSF-secreting melanoma vaccines. *Oncogene*. 2003;22:3188-3192.
30. Sloan JM, Kershaw MH, Touloukian CE, et al. MHC class I and class II presentation of tumor antigen in retrovirally and adenovirally transduced dendritic cells. *Cancer Gene Ther*. 2002;9:946-950.
31. Molldrem JJ. Vaccination for leukemia. *Biol Blood Marrow Transplant*. 2006;12:13-18.
32. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J Immunol*. 1999;163:1690-1695.
33. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909-915.
34. Luznik L, Fuchs EJ. Donor lymphocyte infusions to treat hematologic malignancies in relapse after allogeneic blood or marrow transplantation. *Cancer Control*. 2002;9:123-137.
35. Rooney CM, Aguilar LK, Huls MH, et al. Adoptive immunotherapy of EBV-associated malignancies with EBV-specific cytotoxic T-cell lines. *Curr Top Microbiol Immunol*. 2001;258:221-229.
36. Schwartzentruber DJ, Hom SS, Dadmarz R, et al. In vitro predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J Clin Oncol*. 1994;12:1475-1483.
37. Rosenberg SA. Shedding light on immunotherapy for cancer. *N Engl J Med*. 2004;350:1461-1463.
38. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298:850-854.
39. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol*. 2005;23:2346-2357.
40. Lou Y, Wang G, Lizee G, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. *Cancer Res*. 2004;64:6783-6790.
41. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314:126-129.
42. Hwu P, Shafer GE, Treisman J, et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. *J Exp Med*. 1993;178:361-366.
43. Finer MH, Dull TJ, Qin L, et al. kat: a high-efficiency retroviral transduction system for primary human T lymphocytes. *Blood*. 1994;83:43-50.
44. Stancovski I, Schindler DG, Waks T, et al. Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. *J Immunol*. 1993;151:6577-6582.
45. Daly T, Royal RE, Kershaw MH, et al. Recognition of human colon cancer by T cells transduced with a chimeric receptor gene. *Cancer Gene Ther*. 2000;7:284-291.
46. Kershaw MH, Wang G, Westwood JA, et al. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther*. 2002;13:1971-1980.
47. Maher J, Brentjens RJ, Gunset G, et al. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta/CD28 receptor. *Nat Biotechnol*. 2002;20:70-75.
48. Sakaguchi S. Naturally arising CD4$^+$ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531-562.
49. Shevach EM. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol*. 2000;18:423-449.
50. Rudensky AY, Campbell DJ. In vivo sites and cellular mechanisms of T reg cell-mediated suppression. *J Exp Med*. 2006;203:489-492.
51. Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of CD4$^+$CD25$^+$ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2001;2:301-306.
52. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol*. 2002;3:756-763.
53. Wang RF, Peng G, Wang HY. Regulatory T cells and Toll-like receptors in tumor immunity. *Semin Immunol*. 2006;18:136-142.
54. Wang HY, Lee DA, Peng G, et al. Tumor-specific human CD4$^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity*. 2004;20:107-118.
55. Wang HY, Peng G, Guo Z, et al. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4$^+$ regulatory T cells. *J Immunol*. 2005;174:2661-2670.

56. Chakraborty NG, Chattopadhyay S, Mehrotra S, et al. Regulatory T-cell response and tumor vaccine-induced cytotoxic T lymphocytes in human melanoma. *Hum Immunol*. 2004;65:794-802.
57. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol*. 2004;173:1444-1453.
58. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol*. 2002;129:263-276.
59. Roncarolo MG, Bacchetta R, Bordignon C, et al. Type 1 T regulatory cells. *Immunol Rev*. 2001;182:68-79.
60. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med*. 2003;348:203-213.
61. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.
62. Wolf D, Wolf AM, Rumpold H, et al. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res*. 2005;11:8326-8331.
63. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A*. 2005;102:18538-18543.
64. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol Immunother*. 2002;51:293-298.
65. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol*. 2004;4:941-952.
66. Peranzoni E, Zilio S, Marigo I, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol*. 2010;22:238-244.
67. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med*. 1996;2:1096-1103.
68. Laxmanan S, Robertson SW, Wang E, et al. Vascular endothelial growth factor impairs the functional ability of dendritic cells through Id pathways. *Biochem Biophys Res Commun*. 2005;334:193-198.
69. Gabrilovich DI, Ishida T, Nadaf S, et al. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. *Clin Cancer Res*. 1999;5:2963-2970.
70. Troy AJ, Summers KL, Davidson PJ, et al. Minimal recruitment and activation of dendritic cells within renal cell carcinoma. *Clin Cancer Res*. 1998;4:585-593.
71. Nestle FO, Burg G, Fah J, et al. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am J Pathol*. 1997;150:641-651.
72. Gabrilovich DI, Corak J, Ciernik IF, et al. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res*. 1997;3:483-490.
73. Hoffmann TK, Muller-Berghaus J, Ferris RL, et al. Alterations in the frequency of dendritic cell subsets in the peripheral circulation of patients with squamous cell carcinomas of the head and neck. *Clin Cancer Res*. 2002;8:1787-1793.
74. Della Bella S, Gennaro M, Vaccari M, et al. Altered maturation of peripheral blood dendritic cells in patients with breast cancer. *Br J Cancer*. 2003;89:1463-1472.
75. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol*. 2001;166:678-689.
76. Salvadori S, Martinelli G, Zier K. Resection of solid tumors reverses T cell defects and restores protective immunity. *J Immunol*. 2000;164:2214-2220.
77. Danna EA, Sinha P, Gilbert M, et al. Surgical removal of primary tumor reverses tumor-induced immunosuppression despite the presence of metastatic disease. *Cancer Res*. 2004;64:2205-2211.
78. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J Immunol*. 2001;166:5398-5406.
79. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res*. 2001;61:4756-4760.
80. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol*. 2004;172:989-999.
81. Otsuji M, Kimura Y, Aoe T, et al. Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc Natl Acad Sci U S A*. 1996;93:13119-13124.
82. Schmielau J, Nalesnik MA, Finn OJ. Suppressed T-cell receptor zeta chain expression and cytokine production in pancreatic cancer patients. *Clin Cancer Res*. 2001;7:933s-939s.
83. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res*. 2004;64:5839-5849.
84. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65:3044-3048.
85. Sharma P, Wagner K, Wolchok JD, Allison JP. Novel cancer immunotherapy agents with survival benefit: recent successes and next steps. *Nat Rev Cancer*. 2011;11:805-812.
86. Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. *Clin Exp Immunol*. 2009;157:9-19.
87. Ise W, Kohyama M, Nutsch KM, et al. CTLA-4 suppresses the pathogenicity of self antigen-specific T cells by cell-intrinsic and cell-extrinsic mechanisms. *Nat Immunol*. 2010;11:129-135.
88. Wing K, Yamaguchi T, Sakaguchi S. Cell-autonomous and -non-autonomous roles of CTLA-4 in immune regulation. *Trends Immunol*. 2011;32:428-433.
89. Corse E, Allison JP. Cutting edge: CTLA-4 on effector T cells inhibits in trans. *J Immunol*. 2012;189:1123-1127.
90. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.
91. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
92. Callahan MK, Wolchok JD, Allison JP. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol*. 2010;37:473-484.
93. Wolchok JD, Neyns B, Linette G, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol*. 2010;11:155-164.
94. Robert C, Thomas L, Bondarenko I, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011;364:2517-2526.
95. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
96. Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 1999;11:141-151.
97. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 2001;291:319-322.
98. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol*. 2005;17:133-144.
99. Nomi T, Sho M, Akahori T, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res*. 2007;13:2151-2157.
100. Iwai Y, Ishida M, Tanaka Y, et al. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*. 2002;99:12293-12297.
101. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
102. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
103. Liakou CI, Kamat A, Tang DN, et al. CTLA-4 blockade increases IFNgamma-producing CD4+ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A*. 2008;105:14987-14992.
104. Carthon BC, Wolchok JD, Yuan J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. *Clin Cancer Res*. 2010;16:2861-2871.
105. Khalili JS, Liu S, Rodriguez-Cruz TG, et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. *Clin Cancer Res*. 2012;18:5329-5340.

Drew Pardoll

52

Cancer Immunotherapy with Vaccines and Checkpoint Blockade

Therapeutic vaccination for cancer continues to be a major approach to the overall immunotherapy of cancer. Historically, interest in cancer immunology stemmed from the perceived potential activity of the immune system as a weapon against cancer cells. In fact, the term *magic bullet*, commonly used to describe many visions of cancer therapy, was coined by Paul Ehrlich in the late 1800s in reference to antibodies targeting both microbes and tumors. Central to the concept of successful cancer immunotherapy are the dual tenets that tumor cells express an antigenic profile distinct from their normal cellular counterparts and that the immune system is capable of recognizing these antigenic differences. Support for this notion originally came from animal models of carcinogen-induced cancer in which it was demonstrated that a significant number of experimentally induced tumors could be rejected on transplantation into syngeneic immunocompetent animals.[1-4] Extensive studies by Prehn on the phenomenon of tumor rejection suggested that the most potent tumor rejection antigens were unique to the individual tumor.[5]

Since the original reports of Jenner over two centuries ago, prophylactic vaccination against infectious diseases has been one of the most influential medical interventions. Cancer vaccination, an immunotherapy approach applied to patients with established cancer, has tremendous potential based on the ability of both T cells and antibodies to specifically recognize cancer antigens and kill cancer cells expressing these antigens. However, at the time of this writing, only one human cancer vaccine has received U.S. Food and Drug Administration (FDA) approval, despite multiple Phase III clinical trials over the past two decades. Despite the clinical failures of cancer vaccines to date, continuing molecular definition of tumor-specific and tumor-selective antigens, new vaccine platforms that selectively target and activate dendritic cells, and preclinical results with combinations of vaccination together with other immune modulators have generated renewed optimism that cancer vaccination will ultimately take its place among the pantheon of cancer therapies.

As cancer genetics and genomics have exploded over the past decade, it is now quite clear that altered genetic and epigenetic features of tumor cells indeed result in a distinct tumor antigen profile. Overexpression of "oncogenic" growth factor receptor tyrosine kinases such as HER2/Neu and epidermal growth factor receptor (EGFR) via epigenetic mechanisms has provided clinically relevant targets for one arm of the immune system—antibodies.[6,7] Indeed, monoclonal antibodies are the fastest growing single class of cancer therapeutics based on successful new FDA approvals. In striking contrast, cellular immunotherapy of cancer has been quite disappointing in establishing therapeutic success in clinical trials to date. Emerging insights about the nature of the interaction between the cancer and the immune system have led us to understand why cell-based cancer immunotherapy approaches such as therapeutic vaccines have been less potent against established cancer than originally imagined. In general, we have learned that tumors employ mechanisms of tolerance induction to turn off T cells specific for tumor-associated antigens. Oncogenic pathways in tumors result in the elaboration of factors that organize the tumor microenvironment in ways that are quite hostile to antitumor immune responses.

Not only is the cancer capable of inducing potent tolerance among tumor-specific T cells, we now know that there are distinct forms of inflammatory and immune responses that are procarcinogenic. Thus, two frontiers in cancer immunology are the elucidation of how the tumor organizes its immune microenvironment and the nature of immune responses that are anticarcinogenic versus procarcinogenic. As the receptors, ligands, and signaling pathways that mediate immune tolerance and immune-induced procarcinogenic events are elucidated, these factors and pathways can be selectively inhibited by both antibodies and drugs in a way to shift the balance to antitumor immune responses. This chapter outlines the

major features of tumor–immune system interactions and set the stage for molecularly based approaches to manipulate immune responses for successful cancer therapy. The clinical results over the past few years, particularly with checkpoint blockade, validate clinically the tremendous potential of the immune system to destroy cancer cells (Figure 52-1).

Indeed, adoptive T-cell transfer trials using ex vivo expanded tumor-specific T cells have demonstrated clear proof of the principle that activated tumor-specific T cells can induce tumor regressions, even in patients with bulky metastatic cancer. Because adoptive T-cell transfer is expensive, labor intensive, and extremely difficult to standardize, it is an immunotherapy approach that is difficult to broadly export. Most cancer immunotherapy efforts, including those that involve vaccination, seek to activate and expand tumor-specific T cells in vivo via various manipulations involving standardized reagents. The major barriers to be overcome are induction of tolerance among tumor-specific T cells and a tumor microenvironment that has developed to resist infiltration and attack by activated tumor-specific T cells. Although these two barriers represent significant hurdles to successful cancer immunotherapy, the elucidation of specific molecular mechanisms for tolerance induction as well as immune inhibition within the tumor microenvironment have led to the generation of specific combinatorial approaches to cancer therapy.

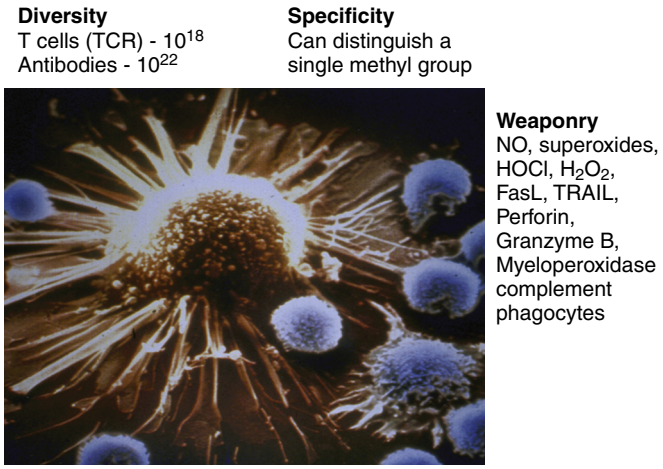


Figure 52-1 The immune system as the perfect anti-cancer weapon Shown in the figure is a single cytotoxic T lymphocyte specific for a tumor antigen expressed by the tumor cell it is about to kill. Although other lymphocytes contact the tumor cell, if they do not express a T-cell receptor specific for a peptide derived from a protein in the tumor cell and presented on MHC class I, they will ignore the tumor cell. Fundamentally, the immune system is endowed with all of the assets desired to specifically eliminate cancer cells while having a minimal effect on normal cells. The tremendous diversity of T-cell receptors and antibodies affords the adaptive immune system both specificity and adaptability. The MHC transport system that carries peptides from degraded proteins to the cell surface allows T cells to recognize protein antigens expressed anywhere in the cell. In addition, the immune system can produce more than 20 cytocidal molecules with diverse mechanisms of killing once activated.

Cancer Antigens—the Difference between Tumor and Self

Tumors reflect the biologic and antigenic characteristics of their tissue of origin but also differ fundamentally from their normal-cell counterparts in both antigenic composition and biologic behavior. Both these elements of cancer provide potential tumor-selective or tumor-specific antigens as potential targets for cancer vaccination specifically and antitumor immune responses in general. Genetic instability, a basic hallmark of cancer, is a primary generator of tumor-specific antigens. The most common genetic alteration in cancer is mutation arising from defects in DNA damage repair systems of the tumor cell.[8-15] Recent estimates from genome-wide sequencing efforts suggest that every tumor contains a few hundred mutations in coding regions.[16] In addition, deletions, amplifications, and chromosomal rearrangements can result in new genetic sequences resulting from the juxtaposition of coding sequences that are not normally contiguous in untransformed cells. The vast majority of these mutations occur in intracellular proteins, and thus the "neoantigens" they encode would not be readily targeted by antibodies. However, the major histocompatibility complex (MHC) presentation system for T-cell recognition makes peptides derived from all cellular proteins available on the cell surface as peptide MHC complexes capable of being recognized by T cells. Based on analysis of sequence motifs, it is estimated that roughly one third of the mutations identified from genome sequencing of 22 breast and colon cancers[16] were capable of binding to common human leukocyte antigen (HLA) alleles based on analysis of sequence motifs.

In accordance with the original findings of Prehn,[5] the vast majority of tumor-specific antigens derived from mutation as a consequence of genetic instability are unique to individual tumors. The consequence of this fact is that antigen-specific immunotherapies targeted at most truly tumor-specific antigens would by necessity be patient specific. However, there are a growing number of examples of tumor-specific mutations that are shared. The three best studied examples are the Kras codon 12 G→A (found in roughly 40% of colon cancers and more than 75% of pancreas cancers), the BrafV599E (found in roughly 70% of melanomas), and the P53 codon 249 G→T mutation (found in about 50% of hepatocellular carcinomas).[17-20] As with nonshared mutations, these common tumor-specific mutations all occur in intracellular proteins and therefore require T-cell recognition of MHC-presented peptides for immune recognition. Indeed, both the Kras codon 12 G→A and the BrafV599E mutations result in "neopeptides" capable of being recognized by HLA class 1– and class II–restricted T cells.[21-24]

The other major difference between tumor cells and their normal counterparts derives from epigenetics.[25] Global alterations in DNA methylation as well as chromatin structure in tumor cells result in dramatic shifts in gene expression. All tumors overexpress hundreds of genes relative to their normal counterparts and, in many cases, turn on genes that are normally completely silent in their normal cellular counterparts. Overexpressed genes in tumor cells represent the most commonly targeted tumor antigens by both antibodies and cellular immunotherapies. This is because, in contrast to most antigens derived from mutation, overexpressed genes are shared among many tumors of a given tissue origin or sometimes multiple tumor types. For example, mesothelin, which is targeted by T cells from vaccinated pancreatic cancer patients,[26] is highly expressed in virtually all pancreatic cancers, mesotheliomas, and most ovarian cancers.[27,28] Although mesothelin is expressed at low to moderate levels in the pleural mesothelium, it is not expressed at all in normal pancreatic or ovarian ductal epithelial cells.

The most dramatic examples of tumor-selective expression of epigenetically altered genes are the so-called cancer-testis antigens.[29] These genes appear to be highly restricted in their expression in the adult. Many are expressed selectively in the testes of males and are not expressed at all in females. Expression in the testis appears to be restricted to germ cells, and in fact some of these genes appear to encode proteins associated with meiosis.[30-32] Cancer-testis antigens therefore represent examples of widely shared tumor-selective antigens whose expression is highly restricted to tumors. Many cancer-testis antigens have been shown to be recognized by T cells from nonvaccinated and vaccinated cancer patients.[29] From the standpoint of immunotherapeutic targeting, a major drawback of the cancer-testis antigens is that none appears to be necessary for the tumors' growth or survival. Therefore, their expression appears to be purely the consequence of epigenetic instability rather than selection, and antigen-negative variants are easily selected out in the face of immunotherapeutic targeting.

A final category of tumor antigen that has received much attention encompasses tissue-specific antigens shared by tumors of similar histologic origin. Interest in this class of antigen as a tumor-selective antigen arose when melanoma-reactive T cells derived from melanoma patients were found to recognize tyrosinase, a melanocyte-specific protein required for melanin synthesis.[33,34] In fact, the most commonly generated melanoma-reactive T cells from melanoma patients recognize melanocyte antigens.[35,36] Although one cannot formally call tissue-specific antigens tumor-specific, they are nonetheless potentially viable targets for therapeutic T-cell responses when the tissue is dispensable (i.e., prostate cancer or melanoma).

From the standpoint of T-cell targeting, tumor antigens upregulated as a consequence of epigenetic alterations represent "self-antigens" and are therefore likely to induce some level of immune tolerance. However, it is now clear that the stringencies of immune tolerance against different self-antigens differ according to tissue distribution and normal expression level within normal cells. The mesothelin antigen described earlier is an example. In a recent set of clinical pancreatic cancer vaccine studies, mesothelin-specific T-cell responses were induced by vaccination with genetically modified pancreatic tumor cell vaccines, and induction of mesothelin-specific T cells correlated with ultimate disease outcome.[37] Given that the immune system is capable of differential responsiveness determined by antigen levels, it is quite possible to imagine generating tumor-selective immune responses against antigens whose expression level in the tumor is significantly greater within normal cells in the tumor-bearing host. In addition, upregulated antigens that provide physiologically relevant growth or survival advantages to the tumor are preferred targets for any form of therapy because they are not so readily selected out.

Beyond the antigenic differences between tumor cells and normal cells, there are important immunologic consequences to the distinct biological behavior of tumor cells relative to their normal counterparts. Whereas uncontrolled growth is certainly a common biological feature of all tumors, the major pathophysiologic characteristics of malignant cancer responsible for morbidity and mortality are their ability to invade through natural tissue barriers and ultimately to metastasize. Both of these characteristics, never observed in nontransformed cells, are associated with dramatic disruption and remodeling of tissue architecture. Indeed, the tumor microenvironment is quite distinct from the microenvironment of normal tissue counterparts. One of the important consequences of tissue disruption, even when caused by noninfectious mechanisms, is the elaboration of pro-inflammatory signals. These signals, generally in the form of cytokines and chemokines, are potentially capable of naturally initiating innate and adaptive immune responses. Indeed, the level of leukocyte infiltration into the microenvironment of tumors tends to be significantly greater than the leukocyte component of their normal-tissue counterparts. Cancers are therefore constantly confronted with inflammatory responses as they invade tissues and metastasize. In some circumstances these inflammatory and immune responses can potentially eliminate a tumor—so-called immune surveillance. However, as discussed later, oncogenic pathways in the tumor appear to organize the immunologic component of the microenvironment in a fashion that not only protects the tumor from antitumor immune responses but can qualitatively shift immune responses to those that actually support and promote tumor growth. It is these elements of

the cancer–immune system interaction that will be the central targets of future immunotherapeutic strategies.

Evidence Pro and Con for Immune Surveillance of Cancer

The fundamental tenet of the immune surveillance hypothesis, first conceived nearly a half century ago,[38,39] is that a fundamental role of the immune system is to survey the body for tumors as it does for infection with pathogens, recognizing and eliminating them based on their expression of tumor-associated antigens. In animal models, carcinogen-induced tumors can be divided into those that grow progressively (termed *progressor tumors*) and those that are rejected after an initial period of growth (termed *regressor tumors*).[1,2] The phenomenon of regressor tumors was thought to represent an example of the ongoing process of immune surveillance of cancer. A corollary to the original immune surveillance hypothesis is that progressor tumors in animals (presumed to represent clinically progressing cancers in humans) fail to be eliminated because they develop active mechanisms of either immune escape or resistance.

A fundamental prediction of the immune surveillance hypothesis is that immunodeficient individuals would display a dramatic increase in tumor incidence. After an extensive analysis of spontaneous tumor formation in immunodeficient nude mice, which have atrophic thymi and therefore significantly reduced numbers of T cells and T-cell–dependent immune responses, no increased incidence of tumors was observed.[40-44] These studies were taken as a major blow to the immune surveillance hypothesis. However, a caveat to the interpretation of these results is that nude mice still produce diminished numbers of T cells via thymus-independent pathways and therefore can mediate some degree of T-cell–dependent immunity. In addition, nude mice frequently display compensatory increases in innate immunity that, as discussed later, may represent a potent form of antitumor immunity and could contribute to immune surveillance of cancer.

Epidemiologic studies of patients with heritable immunodeficiencies revealed a significantly increased risk of certain cancers that are distinct from the epithelial cancers commonly observed in normal immunocompetent adults.[45-47] Many of these cancers are also observed in transplant patients on chronic pharmacologic immune suppression as well as in patients with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) whose immune system is depressed. The most common cancers in these individuals include lymphoblastic lymphomas as well as Kaposi's sarcoma; however, certain epithelial cancers, such as stomach cancer, were also observed at increased frequency. A unifying theme for the majority of cancers observed in immunodeficient individuals is their microbial origin. The majority of lymphoblastic lymphomas are associated with Epstein-Barr virus (EBV),[48] and Kaposi's sarcoma is a result of infection with the herpesvirus KSHV (Kaposi's sarcoma–associated herpesvirus).[49] Other virus-associated cancers such as cervical cancer (from human papillomavirus)[50,51] are also observed at increased frequency. It is now appreciated that stomach cancer is associated with ulcer disease related to infection with the bacterium *Helicobacter pylori*.[52] From these studies, the notion emerged that immune surveillance indeed protects individuals against certain pathogen-associated cancers by either preventing infection or altering chronic infection by viruses and other microbes that can eventually induce cancer. These studies were taken to represent evidence that the common non–pathogen-associated cancers most commonly seen in adults in developed countries (i.e., prostate cancer, colon cancer, lung cancer, etc.) are not subject to immune surveillance.

Two caveats to this interpretation must, however, be noted. First, detailed epidemiologic analyses of immunodeficient individuals were performed at a time when these patients rarely lived beyond their 20s and 30s, when cancer incidence normally increases most significantly. It is therefore possible that a subtler cumulative increased incidence of common non–pathogen-associated cancers would have been observed had these individuals lived further into adulthood. Indeed, more recent analyses definitively demonstrate an increased incidence of some non–pathogen-associated cancers, particularly melanoma, in immunodeficient individuals.[53] In addition to epidemiologic data, dramatic antidotal examples are difficult to ignore. There have been reports that patients receiving kidneys from a cadaver donor who had been in complete remission from a melanoma before organ donation each rapidly developed metastatic melanoma of donor origin after the transplant.[54-56] These results indicate that at least for some non–pathogen-associated tumors, the immune system can play a significant role in maintaining the micrometastatic disease in a dormant state. Whether this principle applies to non–pathogen-associated human tumors besides melanoma remains to be demonstrated.

A number of recent studies reevaluating tumor immune surveillance in genetically manipulated mice has revealed clear-cut evidence that various components of the immune system can at least modify, if not eliminate, both carcinogen-induced and spontaneously arising cancers. A series of studies by Schreiber and colleagues reexamined cancer incidence in mice rendered immunodeficient via genetic knockout of the *RAG2* gene (deficient in both B and T cells), the *γ-interferon receptor* gene, the *STAT 1* gene, or the *type 1 interferon receptor* gene.[57-59] When these knockout mice were either treated with carcinogens or crossed onto

a cancer-prone *P53* knockout background, the incidence of cancers was modestly but significantly increased relative to nonimmunodeficient counterparts when observed over an extended period (longer than 1 year). Transplantation studies demonstrated that direct γ-interferon insensitivity by the developing tumors played a significant role in the defect in immune surveillance. Interestingly, in contrast to *γ-interferon receptor* knockout mice, the mechanism for increased tumor incidence in tumors in *type 1 interferon receptor* knockout mice did not involve sensitivity by the tumor to type 1 interferons but rather reflected role of the type 1 interferons in the induction of innate and adaptive immunity. Even animals not crossed onto a cancer-prone genetic background or treated with carcinogens developed an increased incidence of invasive adenocarcinomas when observed over their entire life span. Furthermore, *γ-interferon, RAG2* double knockout mice developed a broader spectrum of tumors than *RAG2* knockout mice. All of the tumors that arise in these genetically manipulated immunodeficient animals behave as regressor tumors when transplanted into immunocompetent animals. These findings indeed suggest that tumors that arise in immunodeficient animals would have been eliminated had they arisen in immunocompetent animals. The relatively subtle effects on tumorigenesis, requiring observation over the life span of the animal, suggests that the original concept of immune surveillance of tumors arising on a daily basis is in fact not correct. Instead, it is clear that the presence of a competent immune system "sculpts" the tumor through processes that have been termed *immunoediting*.

The immunoediting hypothesis has been somewhat controversial, with differing outcomes in different animal models. One of the caveats in the interpretation of these studies comes from the work of Dranoff and colleagues, who studied mechanisms of increased tumor genesis in GM-CSF × γ-IFN double knockout mice.[60] Although they observed an increase in gastrointestinal and pulmonary tumors, they noted that such animals harbored infection with a particular bacterium not normally observed in immunocompetent animals. Maintenance of these double knockout mice on antibiotics essentially eliminated the increased rate of tumor formation. Thus, it is possible that some of the increased tumor rates in genetically immunodeficient animals could be related to unappreciated chronic infections that develop in these animals, which are not housed under germ-free conditions. Nonetheless, although the classic concepts of immune surveillance of cancer remain unsupported by experimental evidence, studies on tumorigenesis in genetically manipulated immunodeficient mice indeed suggest that developing tumors must actively adapt themselves to their immune microenvironment in order to exist within the context of a competent immune system.

One of the approaches to test the immune surveillance and immunoediting of endogenously arising tumors has been to combine genetically engineered autochthonous tumor models with T-cell receptor transgenic models expressing defined marked T cells specific for a tumor antigen (either the transgenic oncogenic driver protein in the tumors or an antigen co-expressed with the oncogenic driver). In these models, tumor growth can be monitored in immunodeficient versus immunocompetent mice as well as expression of the cognate tumor antigen recognized by the transgenic T-cell receptor. In such a tumor model driven by Kras and p53 loss, tumors emerging in immunocompetent mice either lost antigen or MHC presentation capacity, whereas those emerging in immunodeficient mice did not.[61] In contrast, in a mouse model of spontaneous random oncogene activation, antigen-specific tolerance was generated in immunocompetent mice without evidence for antigen loss.[62] In some models, an intermediate result has been observed. For example, endogenous immune responses can, under some circumstances, establish an equilibrium state with the tumor in which the tumor is prevented from outgrowth in immunocompetent mice but is not completely eliminated.[63] Ultimately, given the model dependence of outcome (i.e., tolerance, versus surveillance versus editing versus equilibrium) it will be important to ascertain which mechanisms are operative in particular human cancers.

Immune Tolerance and Immune Evasion—the Immune Hallmarks of Cancer

Although controversy over the ultimate role of immune surveillance in natural modulation of cancer development and progression will undoubtedly continue into the future, one can summarize the current state of knowledge as supporting the notion that natural immune surveillance plays a much smaller role than originally envisioned by Thomas and Burnet. However, developing tumors need to adapt to their immunologic milieu in a manner that either turns off potentially harmful (to the tumor) immune responses or creates a local microenvironment inhibitory to the tumoricidal activity of immune cells that could inadvertently become activated in the context of inflammatory responses associated with tissue invasion by the tumor. These processes—tolerance induction and immune evasion—have become a central focus of cancer immunology efforts and will undoubtedly provide the critical information necessary for the development of successful immunotherapies that break tolerance to tumor antigens and break down the resistance mechanisms operative within the tumor microenvironment.

Evidence from murine tumor systems as well as human tumors strongly demonstrates the capacity of tumors to induce tolerance to their antigens. This capacity to induce immune tolerance may very well be the single most important strategy that tumors use to protect themselves from elimination by the host's immune system. Tolerance to tumors appears to operate predominantly at the level of T cells; B-cell tolerance to tumors is less certain because there is ample evidence for the induction of antibody responses in animals bearing tumors as well as human patients with tumors. However, with the exception of antibodies against members of the epidermal growth factor receptor family, there is little evidence that the natural humoral response to tumors provides significant or relevant antitumor immunity. In contrast, numerous adoptive transfer studies have demonstrated the potent capacity of T cells to kill growing tumors, either directly through cytotoxic T lymphocyte (CTL) activity, or indirectly through multiple CD4-dependent effector mechanisms. It is thus likely that induction of antigen-specific tolerance among T cells is of paramount importance for tumor survival.

The first direct evidence for induction of T-cell tolerance by tumors was provided by Bogen and colleagues, who examined the response of T-cell receptor (TCR) transgenic T cells specific for the idiotypic immunoglobulin expressed by a murine myeloma tumor.[64,65] They first demonstrated induction of central tolerance to the myeloma protein followed by peripheral tolerance. Using influenza hemagglutinin (HA) as a model tumor antigen, Levitsky and colleagues demonstrated that adoptively transferred HA-specific TCR transgenic T cells were rapidly rendered anergic by HA-expressing lymphomas and HA-expressing renal carcinomas.[66,67] Tolerance induction has been demonstrated in both the CD4 and CD8 compartment. In general, initial activation of tumor-specific T cells is commonly observed; however, the activated state of T cells is typically not sustained, with failure of tumor elimination as a frequent consequence. Tolerance induction among tumor antigen–specific T cells is an active process involving direct antigen recognition, although in some murine systems, tolerance to tumors appears to be associated with failure of antigen recognition by T cells—that is, the immune system "ignores" the tumor.[68,69] Beyond studies on transplantable tumors, more recent analyses of immune responses to tumor antigens in tumor transgenic mice developing spontaneous cancer have further emphasized the capacity of spontaneously arising tumors to induce tolerance among antigen-specific T lymphocytes. In a model of prostate tumorigenesis, Drake and colleagues evaluated CD4 responses to HA and double transgenic animals expressing HA and SV40 T antigen under the control of the prostate-specific probasin promoter.[70] The development and progression of prostate tumors did not result in enhanced activation of adoptively transferred HA-specific T cells. Tolerance to HA as a normal prostate antigen occurred largely through ignorance because there was no evidence for antigen recognition by HA-specific T cells. However, increased recognition was observed on either androgen ablation (which causes massive apoptosis within the prostate) or development of prostate cancer. Nonetheless, enhanced antigen recognition was not accompanied by activation of effector functions such as γ-interferon production. The consequences of transformation in additional tumor transgenic mouse systems have also been analyzed. As described earlier, Blankenstein and colleagues found that preimmunization of mice against the tumor-associated antigen prevented the development of tumors. However, nonimmunized mice developed spontaneous tumors without any significant evidence of natural immune surveillance in the absence of preimmunization. They further demonstrated that an initial antigen-dependent activation of tumor-specific T cells could be observed at the time of spontaneous tumor induction, but that this recognition ultimately resulted in an anergic form of T-cell tolerance similar to that observed by Drake and colleagues in the prostate system.[62]

The capacity of spontaneously arising tumors to tolerize T cells has not been uniformly observed. Ohashi and colleagues observed a contrasting result when LCMV GP33-specific TCR transgenic CD8 T cells were adoptively transferred into double transgenic mice expressing both SV40 T antigen and LCMV GP33 under the control of the rat insulin promoter.[71] These animals develop pancreatic islet cell tumors that express GP33. These investigators found that as tumors progressed in the mice, enhanced T-cell activation occurred. CD8 T-cell activation was demonstrated through bone marrow chimera experiments to occur exclusively via cross presentation in the draining lymph nodes. Despite the activation of tumor-specific T cells, the tumors grew progressively, indicating that the degree of immune activation induced by tumor growth was insufficient to ultimately eliminate the tumors. These results suggest that developing tumors can induce immune responses but may titrate their level of immune activation to one that ultimately does not keep up with tumor progression. Such a circumstance is highly susceptible to the immune editing concept put forward by Schreiber and colleagues in which the tumor edits itself genetically to maintain a sufficient level of resistance to induced immune responses. In the case of the LCMV GP33 T antigen transgenic mice, because neither anergic nor deletional tolerance was observed, animals treated with the dendritic cell stimulatory anti-CD40 antibody demonstrated significant slowing of tumor growth. Thus, it may be possible under some circumstances to shift the balance between tumor immune evasion and tumor immune recognition by agents that affect the overall activation state of either antigen-presenting cells or T cells (see later discussion).

It has been more difficult to obtain definitive evidence that human cancers tolerize tumor-specific T cells, because humans cannot be manipulated the way mice are. However, T cells that are grown out of patients with cancer tend either to be of low affinity for their cognate antigen or to recognize antigens that bind poorly to their presenting HLA (human MHC) molecule, resulting in inefficient recognition by T cells. Recently, a crystal structure of the TCR-peptide-MHC trimolecular complex has been solved for an MHC class II–restricted human tumor antigen.[72] Interestingly, the orientation of the TCR, which has low affinity for the peptide-MHC complex, is distinct from trimolecular complexes for viral (foreign) antigens and is partially similar to trimolecular complexes for a self-antigen. Thus, there may be fundamental structural features of tumor antigen recognition that lie between those of foreign antigen and self-antigen recognition.

As discussed later, one of the features of the tumor microenvironment that is likely central to the capability of tumors to tolerize tumor-specific T cells is the immature or inactive state of tumor-infiltrating dendritic cells (DCs). DCs are the major antigen-presenting cell that presents peptides to T cells to initiate adaptive immune responses. In the context of infection, microbial ligands or endogenous "danger signals" associated with tissue destruction activated DCs to a state in which they present antigens to T cells together with co-stimulatory signals that induce T-cell activation and development of effector function. However, in the absence of microbial products or danger signals, DCs remain in an immature state in which they can still present antigens to T cells but without co-stimulatory signals. These immature DCs function as "toleragenic" DCs, inducing a state of antigen-specific T-cell unresponsiveness (termed *anergy*). It is thought that steady-state presentation of self-antigens by immature DCs is an important mechanism of peripheral self-tolerance. Thus, if a tumor is able to produce factors that inhibit local DCs from becoming activated in response to the endogenous danger signals associated with tissue invasion, it could shift tumor-specific T cells from a state of activation to one of tumor-specific tolerance.

Inhibition of Antitumor Immunity by Regulatory T Cells

Over the past 10 years, regulatory T cells (Tregs) have emerged as a central player in maintenance of the tolerant state as well as general downregulation of immune responses to pathogens.[73,74] Not surprisingly, they appear to play a role in tolerance to tumor antigens as well as the resistance of tumors to immune-mediated elimination.[75,76] In contrast to the ephemeral CD8 suppressor cells of the 1970s that failed to withstand experimental scrutiny, the more recently defined $CD4^+$ Tregs are characterized by expression of a central master regulatory transcription factor—FoxP3—whose role in the gene expression programs of Treg is being actively studied.[77] Although $CD4^+$ Tregs selectively (but not specifically) express a number of cell-membrane molecules, including CD25, neuropilin, GITR, and LAG-3,[78-80] their overall genetic program and inhibitory capacity are absolutely dependent on sustained expression of Foxp3.[81,82] Mechanisms of immune suppression by Tregs vary and include production of inhibitory cytokines such as IL-10 and a recently described IL-12 family "hybrid" cytokine, IL-35, consisting of the alpha subunit of IL-12 and the beta subunit of IL-27.[104] In keeping with the emerging appreciation that tumors are by nature highly tolerogenic, numerous murine studies have demonstrated that Tregs expand in animals with cancer and significantly limit the potency of antitumor immune responses—either natural or vaccine induced. For example, in a study by Sutmuller and colleagues, a combination of GM-CSF transduced tumor vaccine plus anti-CTLA-4 antibodies was much more effective at eliminating established tumors when animals were treated with anti-IL-2 receptor alpha antibodies to eliminate $CD4^+$ Tregs.[83] It is now appreciated that treatment with low-dose cyclophosphamide is a relatively simple and reasonably effective way to temporarily eliminate cycling Tregs.[84-87] This appears to be a major mechanism by which pretreatment with low-dose cyclophosphamide before vaccination can significantly enhance the capacity of vaccines to break tolerance. As new cell membrane molecules that define Tregs are identified, the capacity to block regulatory T-cell activity with antibodies to these molecules presents new opportunities for immunotherapeutic strategies to break tolerance to tumor antigens. Because of their unusually high constitutive expression of high-affinity IL-2 receptors, IL-2 receptor–targeted antibodies and toxin-conjugated IL-2 molecules have been used therapeutically to eliminate Tregs in cancer patients.[88] This continues to be pursued, although its ultimate value will likely come through combination of Treg elimination with other immunotherapy strategies such as vaccination and checkpoint inhibition (see later discussion).

Suppression of Antitumor Immunity by Immature Myeloid Cells in the Tumor Microenvironment

As shown in Figure 52-2, the tumor microenvironment contains multiple inhibitory cells and molecules. Immature myeloid cells (iMCs),[89,90] often termed *myeloid-derived*

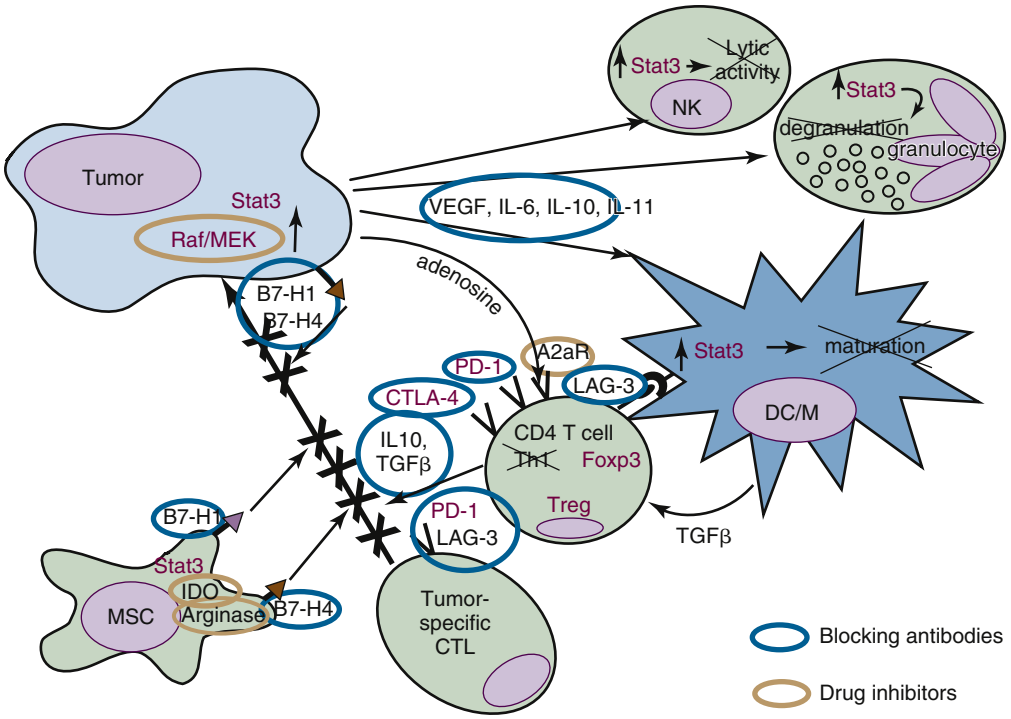


Figure 52-2 The immune microenvironment of a tumor expresses multiple molecules that inhibit immune responses Immune inhibition in the tumor microenvironment is mediated by upregulation of ligands that bind inhibitory receptors on helper T cells and cytotoxic T cells. Multiple immune inhibitory cells, such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg), as well as the tumor, express these inhibitory ligands. In addition, metabolic enzymes, such as IDO, consume nutrients critical for effector T-cell function and produce products inhibitory to T-cell activation. Expression of the molecules is driven by signaling pathways, such as Stat3, that are upregulated in many tumor types. Most of these inhibitory molecules are "druggable" with either small molecules or antibodies.

suppressor cells (MDSCs),[91-94] represent a cadre of myeloid cell types, somewhat overlapping with tumor-associated macrophages (TAMs), which share the common feature of inhibiting both the priming and effector function of tumor-reactive T cells. It is still not clear whether these myeloid cell types represent distinct lineages or different states of the same general immune inhibitory cell subset. In mice, iMCs and MDSCs are characterized by coexpression of CD11b (considered a macrophage marker) and Gr1 (considered a granulocyte marker) while expressing low or no MHC class II or the CD86 co-stimulatory molecule. In humans, they are defined as $CD33^+$ but lacking markers of mature macrophages, DCs, or granulocytes and are DR^-. A number of molecular species produced by tumors tend to drive iMC/MSC accumulation. These include IL-6, CSF-1, IL-10, and gangliosides. IL-6 and IL-10 are potent inducers of STAT3 signaling, which has been shown to be important in iMC/MDSC persistence and activity.

In addition to inhibitory cytokine production, myeloid cells of multiple type in the tumor microenvironment express a number of enzymes whose metabolic activity ultimately results in inhibition of T-cell responses within the tumor microenvironment. These include the production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Nitric oxide (NO) production by iMC/MDSC as a result of arginase and iNOS activity has been well documented, and inhibition of this pathway with a number of drugs can mitigate the inhibitory effects of iMC/MDSC. ROS, including H_2O_2, have been reported to block T-cell function associated with the down-modulation of the ζ chain of the TCR signaling complex,[95] a phenomenon well recognized in T cells from cancer patients and associated with generalized T-cell unresponsiveness.

Another mediator of T-cell unresponsiveness associated with cancer is the production of indoleamine 2,3-dioxygenase (IDO).[96] IDO appears to be produced by DCs either within tumors or in tumor-draining lymph nodes. Interestingly, IDO in DCs has been reported to be induced via backward signaling by B7-1/2 on ligation with CTLA-4.[97,98] The major IDO-producing DC subset is either a plasmacytoid DC (PDC) or a PDC-related cell that is $B220^+$[99]; however, IDO has been subsequently shown to be expressed by multiple cell types in the immune microenvironment, including tumor cells themselves.[100] IDO appears to inhibit T-cell responses through catabolism of tryptophan. Activated T cells are highly dependent on tryptophan and are therefore sensitive to tryptophan depletion. Thus, Munn and Mellor have proposed a bystander mechanism, whereby DCs in the local environment deplete tryptophan via IDO upregulation, thereby inducing metabolic apoptosis in locally activated T cells.[96] IDO has two isoforms, IDO-1 and IDO-2, encoded by distinct genes. The role of IDO-2 in human cancer is still unclear; a major IDO-2 polymorphism in humans encodes an inactive enzyme. A second tryptophan-metabolizing enzyme is TDO (tryptophan dioxygenase), which is upregulated commonly in human cancers—this may inhibit antitumor responses within the microenvironment similarly to IDO.[101] Finally, there has been greater appreciation that a major product of IDO and TDO metabolism of tryptophan—kynurenine—has potent

effects on T-cell differentiation. Under some circumstances, kynurenine can promote Treg development,[102] and under other circumstances, it can promote development of a class of a subset of T cells termed *Th17*,[103] known for its production of IL-17 and for its procarcinogenic properties (see later discussion). Ultimately, the relative role of tryptophan depletion versus kynurenine production in modulating the immune microenvironment remains to be determined.

A major inhibitory cytokine produced by iMC/MSC and by many other cell types that has been implicated in blunting antitumor immune responses is transforming growth factor beta (TGF-β), which is produced by a variety of cell types, including tumor cells, and which has pleiotropic physiological effects. For most normal epithelial cells, TGF-β is a potent inhibitor of cell proliferation, causing cell cycle arrest in the G_1 stage.[104] In many cancer cells, however, mutations in the TGF-β pathway confer resistance to cell cycle inhibition, allowing uncontrolled proliferation. In addition, in cancer cells, the production of TGF-β is increased and may contribute to invasion by promoting the activity of matrix metalloproteinases. In vivo, TGF-β directly stimulates angiogenesis; this stimulation can be blocked by anti-TGF-β antibodies.[105] A bimodal role of TGF-β in cancer has been verified in a transgenic animal model using a keratinocyte-targeted overexpression.[106] Initially, these animals are resistant to the development of early-stage or benign skin tumors. However, once tumors form, they progress rapidly to a more aggressive spindle-cell phenotype. Although this clear bimodal pattern of activity is more difficult to identify in a clinical setting, it should be noted that elevated serum TGF-β levels are associated with poor prognosis in a number of malignancies, including prostate cancer,[107] lung cancer,[108] gastric cancer,[109] and bladder cancer.[110]

From an immunological perspective, TGF-β possesses broadly immunosuppressive properties, and TGF-β knockout mice develop widespread inflammatory pathology and corresponding accelerated mortality.[111] Interestingly, a majority of these effects seem to be T-cell mediated, as targeted disruption of T-cell TGF-β signaling also results in a similar autoimmune phenotype.[112] Recent experiments by Chen and colleagues rather convincingly demonstrated a role for TGF-β in Treg-mediated suppression of CD8 T-cell antitumor responses.[113] In these experiments, adoptive transfer of $CD4^+$ $CD25^+$ Treg inhibited an antitumor CD8 T-cell effector response, and this inhibition was ameliorated when the CD8 T cells came from animals with a dominant negative TGF-β1 receptor.

Beyond inhibitory cytokines and immune inhibitory metabolic enzymes, the therapeutically most relevant inhibitory pathways in the tumor microenvironment are the so-called checkpoints. Immune checkpoints generally refer to membrane ligands that interact with inhibitory receptors on lymphocytes (see later discussion). Many of the checkpoint ligands are upregulated in the tumor microenvironment, either by tumor cells themselves or by myeloid cells within the tumor stroma. The best studies of the checkpoint ligands fall into the B7 family (discussed later), but a number of additional checkpoint ligands do not. Because activated T cells commonly express cognate inhibitory receptors, they are inhibited from mediating antitumor responses, even if they enter the tumor in an activated state. Antibodies against two checkpoint receptors, CTLA-4 and PD-1, as well as against the major PD-1 ligand, PD-L1 (B7-H1), have demonstrated clinical success and are transforming cancer immunotherapy into an accepted cancer treatment modality. These and other checkpoints of potential relevance in tumor immune evasion are covered in the last sections of this chapter.

Therapeutic Cancer Vaccines

Therapeutic vaccines, initially introduced a half century ago, are the most investigated approaches to cancer immunotherapy. Although they are often lumped under one term, the diversity of cancer vaccine formulations is enormous, ranging from cell lysate vaccines, to genetically engineered whole-cell–based vaccines, to dendritic cell vaccines, to peptide- or protein-based vaccines, to engineered viral and bacterial vaccines. The activity in cancer vaccine development translated into many clinical trials beginning in the 1980s and continuing today. As discussed next, newer generations of vaccine design that incorporate scientific principles of dendritic cell biology and T-cell activation are demonstrating more significant clinical benefit after an initial run of failed randomized trials.

Dendritic Cells—The Key Target of Cancer Vaccines

The central theme among cancer vaccination strategies is enhancement of modulation of antigen-presenting cell (APC) function. This is based on the concept that the quantitative and qualitative characteristics of T-cell responses to antigen depend on the signals they receive from the APC. Among the major bone marrow–derived APC subtypes (B cells, macrophages, and dendritic cells), the DC has emerged as the most potent APC type responsible for initiating immune responses.[114,115] As described earlier, DCs associated with cancer have altered properties that result in failure to activate T cells optimally. Cancer vaccines in essence seek to skew the function of DCs toward the generation of effector T-cell responses.

As virtually all phases of DC differentiation and function can be modulated by engineered vaccines, it is important to understand the molecular signals that regulate their role in activation of T-cell–dependent immunity. At sites of infection and inflammation, bone marrow–derived progenitor cells respond to both proliferative and differentiation signals. GM-CSF and other cytokines such as FLT-3L and IL-4 serve as mitogenic or comitogenic factors that induce an intermediate stage of DC differentiation, characterized by efficient antigen uptake and processing.[116-120] Once they have ingested antigens at inflammatory sites in the tissue, immature DCs differentiate in response to a number of distinct "maturation" signals. Although many diverse molecules induce DC maturation, most appear to signal DCs via binding to two classes of receptors—the Toll-like receptors (TLR) and the TNF receptor (TNFR) family. TLRs are "pattern recognition receptors" (PRR), which bind common chemical moieties expressed by pathogens termed *pathogen-associated molecular patterns* (PAMPs) such as lipopolysaccharide (LPS) and unmethylated CpG DNA sequences.[121] The two best characterized endogenous DC maturation factors are TNF-α itself and CD40L.[122-124] In addition to TLRs, intracellular PRRs, including PKR, RIGI, MDA-5, and NOD1/2, recognize PAMPs from intracellular bacteria and viruses that invade the cytosol.[125-127]

Maturation of DCs, which occurs as they traffic to draining lymph nodes, is characterized by transport of peptide-MHC complexes to the cell surface.[128,129] In addition to provision of high densities of peptide-MHC complexes for T-cell stimulation (termed *signal 1*), DCs regulate T-cell activation and differentiation through the provision of co-stimulatory signals in the form of cytokines, such as IL-12, and membrane-bound ligands of the B7 and TNF family (collectively termed *signal 2*). The ever-expanding panoply of co-stimulatory signals used by DCs to instruct T cells as to their pathway of differentiation and effector function defines a high degree of complexity to the communications that occur between APC and T cell. When immature DCs present antigens to T cells in the absence of co-stimulatory signals, the outcome is tolerance induction (Figure 52-3). This is a normal mechanism for the maintenance of tolerance to self-antigens. It is also a mechanism by which tumors can induce immune tolerance to their own antigens. As discussed throughout this chapter, tumor-induced immune tolerance is a major barrier to successful vaccination of established cancers. Each of the molecular events involved in proliferation, antigen presentation, and co-stimulation represents potential targets that are being exploited in the design of immunotherapy approaches.

Prior to the molecular identification of tumor-specific antigens, investigators used tumor cells themselves as a source of tumor antigen (Table 52-1). Efforts to modify

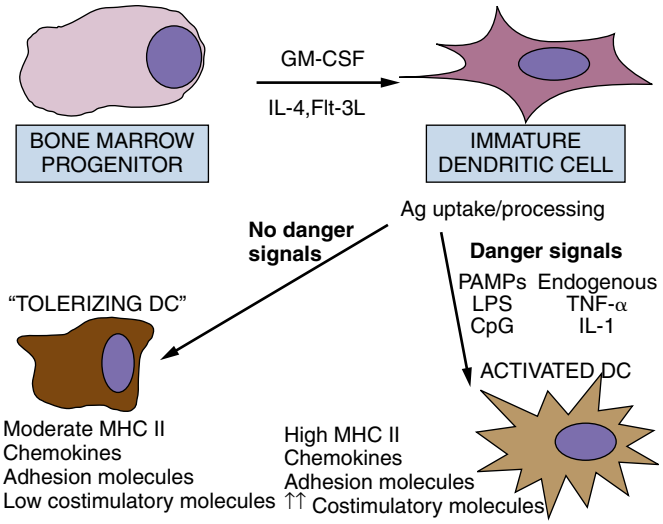


Figure 52-3 Dendritic cells can mediate immune tolerance or immune activation Dendritic cells (DCs), the sentinels of the immune system, develop from hematopoietic progenitors under the influence of cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF). In the presence of microbial infections, certain microbial products such as lipopolysaccharide (LPS)—often called danger signals—bind sensing molecules on the DC surface, phagosome or cytosol. This induces expression of high levels of MHC molecules and co-stimulatory molecules that aid in activation of T cells specific for ingested antigens. In the absence of these danger signals, DCs can also present antigens to T cells, but in the absence of co-stimulatory molecules. These DCs also express high levels of co-inhibitory molecules for so-called checkpoint receptors on T cells. The result is that T cells become refractory to activation, a state referred to as *anergy*. In some cases, anergic T cells die. Either of these fates generates immune tolerance specific for those antigens.

tumor cells as vaccines date back roughly half a century. Whole-cell tumor vaccines have been generated through mixing with adjuvants aimed at enhancing "immunogenicity" of tumor-specific or tumor-selective antigens incorporated therein, with clinical testing of these mixtures dating back to the 1980s.[130-134] Another approach has been to hapten modify whole tumor cells with chemicals such as dinitrophenol[135] or infect them with a virus.[136] The general concept is that increasing the immunogenicity of tumor cells using either adjuvants or expression of foreign antigens will enhance immune responses to the endogenous tumor antigens, thereby allowing the immune system to kill metastatic tumor deposits.

More recently, a new era in genetically engineered whole-cell vaccination has involved the modification of tumor cells through the transfer of genes encoding cell membrane immunostimulatory molecules or cytokines. Although most of the clinical activity related to adjuvanted whole-cell vaccines is diminishing significantly, active clinical investigation continues for cytokine gene-modified whole-cell vaccines, particularly with the GM-CSF gene (described later). Adjuvanted whole-cell tumor vaccines have been tested extensively in patients with melanoma, renal cell carcinoma, and colorectal carcinoma. Most of these vaccine strategies have

Table 52-1 General Formulations for Cancer Vaccines

Vaccine Type	Advantages	Disadvantages
Cell-based vaccines		
Autologous Allogeneic	Highly polyvalent antigenic content Vaccine antigens match those of patient's tumor Generic vaccine for all patients	Contains mostly self-antigens Individualized patient formulation Relies solely on shared antigens
Dendritic cell (DC)		
Peptide loaded Protein loaded Tumor lysate loaded RNA transduced	Can manipulate DC type and direct Ag loading Specific antigen and easy to synthesize Specific antigen and no need for HLA match Highly polyvalent antigenic content Can amplify from tiny amount of tumor	Individualized cell culture Must match patient's HLA type Loading of HMC I less efficient Contains mostly self-antigens Complex individualized process
Peptide + adjuvant	Very easy to produce	Limited immunogenicity
Protein + adjuvant	Moderately easy to produce	Potency adjuvant-dependent
DNA vaccine	Easy to produce, versatile construction	Limited immunogenicity
Viral vaccine	Moderately easy to produce, immunogenic	Neutralizing Ab limit revaccination
Bacterial vaccine	Easy production, immunogenic, incorporate many Ag	Regulatory challenges

involved the co-injection of either autologous or allogeneic tumor cells with adjuvants such as bacille Calmette-Guérin (BCG) and *Corynebacterium parvum*.[130-134,137] Although BCG and *C. parvum* were long known to represent reasonable vaccine adjuvants for the generation of antibody responses, a limitation of this vaccination approach has been their relatively poor capacity to generate T-cell responses, particularly in the face of established tolerance. Initially, nonrandomized clinical trials were performed that demonstrated hints of promise. In some of these studies that reported antitumor responses, the responses were shown to correlate with the return of delayed-type hypersensitivity (DTH) responses to recall antigens and more importantly with the development of DTH responses to autologous tumor cells.

The application of BCG-adjuvanted tumor cell vaccines to patients with bulky metastatic cancer demonstrated an insignificant clinical response rate. However, given the plethora of studies in animal models suggesting that cancer vaccination might be more effective in the setting of minimal residual disease, a number of studies employing BCG-adjuvanted tumor vaccines in clinical trials were undertaken in the minimal residual disease setting after resection of the primary tumor. Initial enthusiasm for a BCG-adjuvanted, autologous colon cancer vaccine in patients with resected stage II/III colon cancer[138] as well as a melanoma vaccine consisting of a mixture of irradiated allogeneic human melanoma lines with BCG used in melanoma patients with stage III and resected stage IV disease[139] was based on Phase II studies and limited single-institution Phase III studies. The concern in the interpretation of clinical outcomes of these Phase II studies is that it was unclear whether the untreated "historical controls" were truly comparable to the population of patients treated in the Phase II studies. In the absence of careful case-controlled comparisons, the ultimate acceptance of these vaccines depended on pivotal randomized Phase III studies in which both progression-free survival and overall survival were the relevant clinical endpoints. In the case of the autologous BCG-adjuvanted colon cancer vaccine, an initial randomized single-institution study in The Netherlands claimed a longer overall survival in patients with stage II but not stage III colon cancer.[140] Unfortunately, these findings were not reproduced in expanded multicenter trials, possibly owing in part to technical difficulties in consistent autologous tumor preparation as part of the patient-specific vaccine formulation.[141] After 20 years of Phase I and II studies with an allogeneic BCG-adjuvanted melanoma vaccine, a randomized Phase III clinical trial of BCG-adjuvanted allogeneic melanoma cells versus BCG control demonstrated no evidence of enhanced overall survival for the BCG plus tumor vaccine arm.[142] Although the Phase II studies claimed to have demonstrated significant survival benefit relative to case-matched controls, the case-matched controls demonstrated suspiciously short overall survival times relative to melanoma patients of similar stage from multiple other clinical studies. There were encouraging reports of responses to vaccination with BCG-adjuvanted DNP-modified allogeneic melanoma vaccines.[143] However, definitive randomized Phase III trials have not been completed at the time of this writing. Although a number of these studies reported that patients with enhanced DTH responses post vaccination had better disease outcomes than patients who did not, these studies were largely devoid of analyses of antigen-specific T-cell responses, and it is unclear whether the association between DTH responses and enhanced survival had anything to do with the vaccination. A similar fate befell the melanoma vaccine Melacine, a mixture of lysates from

multiple allogeneic melanoma cells admixed with the "detoxified" LPS derivative monophosphoryl lipid A (MPL) plus mycobacterial cell wall extracts. Despite encouraging reports from Phase II studies, a definitive Phase III study in patients with stage II/III operated melanoma failed to demonstrate a statistically significant effect on overall survival.[144] A retrospective subset analysis suggested that HLA-A2$^+$ and HLA-C1$^+$ patients had greater benefit, but this result has not been confirmed in a prospective trial.

One of the limitations of these trials is that none demonstrates definitive enhancement of T-cell responses against relevant antigens. In the case of melanoma, many tumor antigens recognizable by T cells are indeed well defined and responses to them should be measured as part of the development process. As described earlier, a more limited set of "immunorelevant" antigens are defined for other human cancers. In summary, the age of adjuvanted whole-cell or lysate tumor vaccines appears to be slowly drawing to a close and will likely be a historical footnote in the development of cancer immunotherapies.

Genetically Modified Tumor Vaccines

With the development of improved genetic techniques, emphasis shifted to genetic modification of tumor cells to express immunostimulatory molecules. Building on the original studies of Lindenmann and Klein,[145] who showed that vaccination with influenza virus–infected tumor-cell lysates generated enhanced systemic immune responses against challenge with the original wild-type tumor, Fearon and colleagues use direct gene transfer to introduce the immunogenic influenza hemagglutinin (HA) gene into murine tumor cells to create genetically engineered vaccines.[146] These HA transfectants induced a systemic immune response against challenge with the parental tumor. Gene transfer of viral antigens was eventually superseded by gene transfer of immune response–modulating genes. It is important to point out that although many of the strategies were designed with a specific mechanism in mind, it is becoming clear that genetic manipulation to alter expression of even a single gene product can result in a complex cascade of cellular responses in vivo that ultimately may affect multiple aspects of antigen processing, presentation, and co-stimulation.

There are many ways to genetically modify tumor cells to augment T-cell–mediated antitumor immunity. One involves the genetic modification of tumor cells to express cytokines that function as attractants or differentiating agents for dedicated APCs such as dendritic cells.[147] Recruited DCs ingest released tumor antigens at the site of vaccination and present them together with appropriate co-stimulation required for the activation of a tumor-specific T-cell response. Alternatively, the tumor cell can be genetically modified such that it becomes the APC itself.

Both ex vivo and in vivo methods of gene delivery have been employed in the development of genetically modified whole-cell cancer vaccines. Ex vivo gene delivery involves the modification of cultured cells. The genetically modified cells are subsequently administered to the host, typically after irradiation. Clearly, the most effective way to enhance expression of MHC molecules or to enhance expression of co-stimulatory molecules such as B7.1 or B7.2 is to genetically modify the tumor cell itself. However, when the goal is to deliver cytokines locally in a paracrine fashion, genetic modification of the tumor cells themselves is not necessary. A number of transduced bystander cytokine delivery systems have been developed.[148] The efficacy of bystander cytokine delivery systems is comparable to that of direct gene modification of the tumor cell for augmenting antitumor immunity. It is, however, necessary that the transduced bystander cells be admixed with the tumor cells in an appropriate ratio.

Genes that encode cytokines are the most common types of genes that have been introduced into tumor cells in order to generate genetically modified tumor vaccines.[147] Tumor cells transduced with cytokine genes alter the local immunologic environment at the vaccine site, enhancing either the presentation of tumor-specific antigens to the immune system or the activation of tumor-specific lymphocytes. Critically, the cytokine is produced at very high concentrations in the vicinity of the tumor, whereas systemic concentrations are relatively low. This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines. Since the initial reports of enhanced antitumor responses after vaccination with IL-2-transduced tumor vaccines,[149,150] many cytokine genes have been introduced into tumor cells with various effects on both tumorigenicity and immunogenicity. Some of these cytokines induce a local inflammatory response that results in elimination of the injected tumor. This local inflammatory response is predominantly dependent on components of innate immunity rather than the classic T cells. Ultimately, however, the most important outcome of vaccination is the generation of enhanced T-cell responses specific for the antigens expressed by the vaccinating tumor.

Among the vast array of cytokine gene–transduced tumor vaccine studies, GM-CSF–transduced tumor vaccines remain as the most actively pursued clinically, despite their recent failure in Phase III trials in prostate cancer. In the original study that identified GM-CSF, multiple cytokine, adhesion molecule, and co-stimulatory genes were introduced into the poorly immunogenic B16-F10 tumor using a replication-defective retroviral vector that produced consistent high levels of expression of each of the transgenes in the absence of

selection, thereby eliminating variability caused by different levels of gene expression and resultant cytokine expression. Animals were vaccinated with the irradiated transductants, followed by challenge with unirradiated wild-type B16-F10 cells to doses 3-4 logs higher than the minimal tumoricidal dose.[151] Although a number of cytokine genes in that study, such as IL-4 and IL-6, induced some measurable systemic antitumor immunity,[152,153] the most potent systemic antitumor effect was produced by GM-CSF–transduced tumor cells. Many subsequent studies in other murine tumor models have validated the potent systemic immunity induced by GM-CSF–transduced tumor vaccines. Antitumor immunity induced by GM-CSF–transduced vaccines has been shown to depend on $CD4^+$ and $CD8^+$ T cells. In addition to the classic MHC class I–restricted CTLs, other effector arms mediated by CD4 cells have been shown to participate in the generation of maximal antitumor immunity. TH1 and TH2 effector arms have been delineated.[154] The TH1 effector arm depends on IFN-γ and involves the activation of macrophages at sites of metastases to produce reactive nitrogen species such as NO, as well as reactive oxygen species (superoxides). Eosinophils appear to be important TH2 effectors that are dependent on the production of cytokines such as IL-4 and IL-5 by tumor-specific CD4 cells. The presence of eosinophils at DTH sites and in tumor metastases subsequent to vaccination with GM-CSF–transduced tumors is not only observed in animal models, but also has been a consistent observation in clinical trials with different tumor types.[155]

Clinically, non–patient-specific GVAX platforms that use GM-CSF–modified allogeneic tumor cells exclusively offer numerous advantages over their autologous cell counterparts including manufacturing issues, consistency of vaccine, and fewer limitations on vaccine quantity. Scientific data from both preclinical and clinical studies have provided support for the relevance of allogeneic GVAX immunotherapies and the seminal role of cross presentation of allogeneic tumor-associated antigens by host APCs in the initiation of a cellular antitumor immune response. The one drawback of allogeneic GVAX vaccines is that unique, patient-specific tumor antigens are not targeted.

Antigen-Specific Vaccines

The ultimate goal of cancer vaccine development is the use of antigen-specific vaccines that incorporate select tumor antigens into a vaccine vector(s) or adjuvanted formulation(s) (see Table 52-1). Antigen-specific vaccines have two intrinsic advantages over any type of cell-based vaccine. First, their formulation into a vaccine is much more versatile. Second, they do not contain the thousands of irrelevant or autoantigens included in cell-based vaccines. The control over antigenic makeup afforded by antigen-specific vaccines is significant but requires knowledge of the best antigens to incorporate. An ideal tumor antigen: (1) should be highly selectively expressed by the tumor relative to normal tissues and expressed at reasonably high density on the surface of the tumor cell (as peptide-MHC complexes for T-cell recognition); (2) should be shared among the majority of tumors of a particular type or ideally tumors with diverse histologies; (3) should provide a growth advantage for the tumor, ideally required for tumor growth or survival; and (4) should have a reasonable T-cell repertoire available in patients that has not been deleted and is not stringently tolerized. One of the major limitations in many of the antigen-specific vaccines tested clinically has been the application of antigens that do not meet these criteria. No one antigen may exist that does perfectly meet all these criteria. However, when planning to test a vaccine clinically, it is important to ask how well the candidate antigen(s) meet them. Ideally, antigen-specific vaccines should contain multiple "immunorelevant" antigens, particularly if they are not absolutely essential for tumor growth or survival. A final general principle is that a vaccine containing the best tumor antigen(s) will not enhance antigen-specific responses effectively if the vaccine vector or adjuvant is suboptimal. These principles will be evaluated in the later discussion of commonly studied antigen-specific vaccines.

Peptide Vaccines

The identification of T-cell–recognized tumor antigens at the peptide level spawned a major effort beginning in the 1990s to develop peptide vaccines.[156-158] The fundamental concept of peptide vaccination is that minimal peptides—particularly MHC class 1–restricted peptides that are recognized by CD8 killer cells—can efficiently load MHC molecules on the surface of cells without requiring internal antigen-processing routes. Early studies with peptide vaccines mixed with various adjuvants demonstrated the induction of peptide-specific T cells in vivo and in some cases antitumor responses.[159]

Clearly, one of the major advantages of peptide vaccines is that they represent the ultimate defined tumor antigen, and therefore the capacity to monitor induction of T-cell responses to the immunizing peptide is optimal. However, there are a number of disadvantages associated with peptide vaccination. First, individual peptides are selective for specific MHC alleles and therefore cannot be used generically. This limitation has been circumvented through the use of mixtures of peptides that bind to common MHC alleles, thereby ensuring that the vast majority of patients will express at least one MHC allele that can present the peptide in the vaccine mix. Another major issue with

minimal peptides as vaccinating antigens is that they not only load the MHC molecules of DCs that would activate immune responses, but also bind to MHC molecules on the surface of cells other than DCs just as efficiently. The consequences of peptide presentation by these cells can be tolerance induction because they do not supply the appropriate co-stimulatory signals necessary for T-cell activation.[160-162] Therefore it is possible that peptide vaccination could in fact be detrimental for immune responses. Indeed, Melief and colleagues have presented evidence in animal models that vaccination with long peptides that require processing is significantly superior to vaccination with minimal MHC binding peptides.[163] They have demonstrated that the advantage of vaccination with long peptides comes from the fact that only DCs can process long peptides, thus leading to selective antigen presentation by DCs over other APCs that could induce tolerance.

Another major factor in peptide vaccinations is the adjuvant that is used. Peptides themselves are intrinsically nonimmunogenic, and strong immunization with peptides in animal models is observed only when strong adjuvants capable of activating dendritic cells are mixed with the peptides. Peptides can also be loaded onto DCs grown ex vivo (see later discussion) and reinjected into the patient. The most common formulation used for peptide vaccines in clinical trials is incomplete Freund's adjuvant (IFA), an oil emulsion that does not contain any specific activators of DCs and is thus suboptimal. A few groups have reported enhanced immunogenicity of peptides conjugated to lipids (lipopeptide vaccines).[164,165]

Clinical trials with peptide vaccines in cancer have predominantly used HLA class I–restricted tumor antigen peptides, but some are including MHC class II–restricted peptides.[166] The inclusion of MHC class II–restricted peptides can involve either peptides derived from tumor antigens (such as tyrosinase in the case of melanoma), or those derived from foreign antigens that would nonspecifically stimulate CD4 helper cells, which theoretically would provide help for enhanced stimulation of tumor-specific CD8 T cells responding to the tumor-specific MHC class I–restricted peptides.

Clinical trials have been performed using peptide vaccines for many different cancer types, though vaccination for melanoma is the most common clinical target of peptide vaccines. A number of clinical trials using peptides either in the setting of bulky metastatic cancer or in the minimal residual disease setting have demonstrated induction of increased numbers of antigen-specific T cells using various methods with anecdotal clinical responses.[167,168] Some methods use staining with peptide-MHC tetramers to directly visualize antigen-specific T cells. Other methods such as ELISPOT or intercellular cytokine staining (ICS) seek to measure induction of functional T cells through the production of cytokines such as γ-interferon.

Among the most interesting clinical results highlighting the dichotomy between induction of expanded numbers of peptide-specific T cells and the absence of clinical activity are the vaccine trials in melanoma that have used an anchor-modified gp100 peptide to generate enhanced binding to HLA-A2.[169] These trials use repetitive vaccination with this peptide in IFA in patients with no evaluable disease (NED) after resection for stage II to stage IV melanoma. In these trials, Rosenberg and colleagues demonstrated the capacity to induce tremendous expansion of antigen-specific CD8 T cells, in some patients reaching 50% of the total circulating CD8 T cells as measured by staining with peptide-MHC tetramers and peptide-induced γ-interferon production. Nonetheless, there was no evidence that relapse rate was significantly different from the relapse rate in the same group of melanoma patients not receiving vaccination. In some cases, relapsed melanomas could be demonstrated to have lost HLA-A2 expression or expression of the gp100 antigen, possibly representing an example of evasion or escape from the T-cell responses induced by vaccination. However, many of the relapsed tumors expressed HLA-A2 and gp100, thereby suggesting that the expanded populations of HLA-A2/gp100-specific T cells induced by peptide vaccination were ineffective at eliminating relapsing tumors. Rosenberg and colleagues recently summarized the clinical experience with peptide vaccines, indicating that peptide vaccines as single agents in the advanced disease setting provide a meager 2% to 3% objective response rate.[170] However, more recent clinical studies, adding various adjuvants to peptide vaccines, mixing multiple MHC class I and MHC class II–restricted tumor peptides together,[171] and using "long peptides,"[172] suggest that the maximal potential benefits of peptide vaccines have yet to be realized. As more is learned about the regulation of T-cell responses, it is quite plausible to imagine that T cells expanded in suboptimal conditions (i.e., in the absence of appropriate proinflammatory or co-stimulatory signals) could upregulate expression of inhibitory molecules that would block them from developing the critical effector activity necessary to kill tumor cells. Thus, tumor antigen–specific cells could expand but not be effective against tumors.

Ex Vivo Loaded DC Vaccines

The ability to culture DCs ex vivo has led to a plethora of studies of ex vivo antigen-loaded DCs as tumor vaccines. Although DCs can be loaded with lysates of tumor cells, they are typically either loaded with peptides or recombinant protein or transduced with various vectors or RNA encoding specific antigens. Initially, it was demonstrated that loading of ex vivo cultured DCs with MHC class

I–restricted peptides, whole proteins, or tumor lysates followed by administration back into the animal led to the generation of immune responses against the loaded antigen as well as antitumor responses.[173-179] More recently, the advent of more efficient gene transfer vectors has led to approaches in which ex vivo cultured DCs are transduced with genes encoding relevant viral or tumor antigens.[180-182] A number of recombinant replication-defective viruses have been used to transduce DCs. In addition, Gilboa and colleagues have demonstrated that purified RNA can be used to effectively transduce DCs with resultant presentation of encoded antigens.[183] This strategy offers the interesting possibility that DCs could be transduced with the entire amplified transcriptome of a tumor cell, even when only tiny amounts of tumor tissue are available. At present, the paucity of direct comparative studies leaves open the question of which method of loading DCs ex vivo will be the most effective. Another major issue with ex vivo loaded DC vaccines is the degree of maturation that is induced in vitro and its relevance to homing and function of loaded DCs after reinjection. Maturation protocols used for DC vaccination are currently quite variable and range from monocyte conditioned medium to various defined agents such as TNF-α, IL-1, soluble CD40L, and prostaglandins.[184,185] Concern has been raised that full-blown maturation/activation of DCs ex vivo to a stage normally achieved once they are within paracortical regions of the lymph node will impair their ability to home to lymph nodes after reinjection. This has led to the suggestion that DCs should be loaded and reinjected in an immature state and allowed to mature in vivo. However, such an approach has potential negative consequences, as Steinman, Bhardwaj, and colleagues have demonstrated—immunization of patients with antigen-loaded immature DCs can actually result in tolerance or suppression of antigen-specific responses.[186]

Elucidation of proliferative and maturation signals for DCs has led recently to approaches in which DCs not only are loaded with antigen but are transduced with genes encoding proliferation and maturation signals. This would result in autocrine DC stimulation in vivo after reinjection. In one study, DCs loaded with antigen were transduced with genes encoding GM-CSF and CD40L. These genetically modified DCs resulted in much more potent stimulation of antitumor immunity than immunization with DCs loaded with antigen alone.[187]

Another approach aimed at providing DCs with a full complement of tumor antigens is the generation of DC-tumor fusion vaccines.[188-190] The concept behind this approach is to fuse autologous tumor cells with dendritic cells, thereby allowing for the coexpression of all relevant tumor antigens together with all relevant DC molecules within the same cell. One of the major limitations to clinically translating an approach of this type is the efficiency with which fusion can be achieved between DCs and tumor cells in the absence of selection. Ultimately, it is critical that both preclinical and clinical DC vaccine studies identify the critical parameters of DC growth and maturation as well as antigen loading that result in therapeutically relevant levels of T-cell activation in vivo.

Many clinical trials with DC vaccines have been performed using DCs cultured and activated in vitro by various methods and loaded with tumor antigens of various types. As with most cancer vaccines, melanoma is the most common target, although other cancers have been targeted as well. Induction of T-cell responses is commonly reported, and interesting anecdotal clinical responses have been reported in Phase I/II trials.[117] One of the more interesting clinical DC vaccine approaches involves transduction of DCs with RNA encoding telomerase that is targeted to the MHC class II processing pathway with the LAMP targeting signal.[191] Telomerase is the enzyme that restores telomeres, the ends of chromosomes. Without telomerase, cells will eventually stop growing when their telomeres are exhausted. Tumors typically upregulate telomerase, making it a tumor-selective antigen. Vaccination with DCs transduced with telomerase-LAMP RNA led to significantly enhanced CD4 and CD8 responses specific for telomerase.

Two Phase III clinical trials using DC vaccines have been negative, though one led to interesting politically motivated deliberations at the U.S. FDA. Schuler and colleagues compared DTIC chemotherapy with peptide-loaded DC vaccination in stage IV melanoma patients.[192] Objective responses were low in both arms (less than 5%), and there was no statistically significant difference in overall survival. A retrospective subset analysis suggested that HLA-$A2^+$/HLA-$B44^-$ patients might derive greater benefit from vaccination, but this has not been verified in a prospective manner. The Dendreon Corporation has recently reported results of a Phase III trial in patients with advanced prostate cancer comparing placebo with a DC vaccine prepared by crude enrichment of peripheral blood lymphocytes followed by culture with a prostatic acid phosphatase–GM-CSF fusion protein.[193] The primary endpoint of prolonged progression-free survival was not achieved, but continued evaluation of the patients demonstrated prolonged overall survival compared with placebo of 4.5 months. The quality of this trial was questionable because the small size precluded careful matching of patient characteristics and registration was applied for based on an endpoint different from that built into the original trial. In addition, the initial evaluation of a follow-up Phase III trial did not demonstrate even a significant trend toward improved overall survival in the DC-vaccinated group. Although a majority of the advisory

panel voted to approve the vaccine, the FDA ultimately chose to require additional supporting clinical data before approval.[194] However, a follow-up Phase III trial with overall survival as the endpoint has recently been reported to have met its endpoint (see later) and, at this writing, is in the process of Biologics License Application (BLA) filing.

Heat Shock Protein–Based Vaccines

Another interesting category of proteins that may target antigen effectively to DCs and furthermore into MHC processing pathways are the heat shock proteins (HSPs). It is now well established that complexing peptide antigens to certain HSPs such as gp96, hsp-70, calreticulin, and hsp-110 enhances their immunogenicity significantly.[195-202] HSPs were first used as tumor vaccines by purifying them from tumor cells followed by immunization. HSPs isolated from tumors are naturally complexed with a whole array of tumor-associated peptides. Other approaches to link antigen to HSP have included the production of recombinant fusion proteins in which antigenic peptides are covalently or noncovalently linked to the HSP[203] as well as DNA-based vaccines in which fusion genes between antigen and HSP gene are incorporated. In one direct comparative study using the human papillomavirus (HPV)-E7 antigen as a model, it was demonstrated that DNA vaccines encoding an E7-hsp70 fusion gene were 30-fold more effective than the wild-type E7 gene in generating $CD8^+$ responses.[204] Immunogenic HSPs complexed with antigenic peptides have been shown to efficiently load the MHC class I processing pathway (so-called in vitro cross presentation).[205] Although the intracellular pathway by which HSPs effectively load MHC class I molecules with their associated peptides has not yet been elucidated, Srivastava and colleagues have identified CD91, the α2 macroglobulin receptor, as an important receptor for several HSPs (gp96, hsp-70, hsp-90).[206] Ultimately, the immunogenicity of HSPs has been proposed to result from their ability to activate APCs and target antigens to MHC processing pathways. One report has suggested that hsp-70 can activate macrophages via CD14/TLR-4 (LPS receptor)–dependent and –independent pathways.[207] HSPs have also been reported to activate DCs,[208] although the receptors that mediate these putative activation functions have yet to be elucidated.

Clinical trials with HSPs have been ongoing. A Phase II trial vaccinating women with premalignant high-grade cervical dysplasia caused by HPV-16 using a bacterial hsp65-HPV16 E7 fusion protein demonstrated a 35% CR with induction of E7-specific T-cell responses in roughly half of the patients; however, the cohort was too small to determine whether this response rate was statistically different from the roughly 25% spontaneous regression rate observed in this patient group without treatment.[209] The only Phase III trial with HSP vaccines reported to date was in patients with operated stage II/III renal cancer, who were randomized to observation or treatment with autologous hsp96 purified from the resected primary tumor. This was a negative study in that no statistical difference was observed in either relapse-free survival or overall survival. A second Phase III trial of autologous hsp96 versus physician's choice (IL-2, resection, or chemotherapy) demonstrated no benefit relative to the physician's choice arm but found that patients receiving 10 or more vaccine administrations had a longer overall survival than those who received fewer administrations of vaccine.[210]

The Growing Armamentarium of Vaccine Vectors

For all of the added value that recombinant DNA technology provides in engineering elements into vaccine constructs that enhance their potency, nature itself provides a virtually limitless array of delivery systems in the form of diverse microbes with potent intrinsic immunologic properties. These immunogenic properties derive from their expression of PAMPs, which activate DCs via TLRs and intracellular sensing pathways such as PKR, RIGI, and MDA-5; their ability to induce proinflammatory cytokine expression by infected cells; and their ability to target intracellular MHC processing compartments. Of the three major microbial classes—virus, bacterium, and fungus—viruses and bacteria have been the most intensively investigated. A few reports of engineered yeast vaccines emphasize the potential immunologic utility of the third microbial class.

Engineered Viruses

Viruses are the most diverse and efficient gene transfer agents whose natural cell tropism and biologic features can significantly enhance the immunogenicity of antigens carried within them (see Table 52-1). Using standard recombination approaches, Moss and Paoletti were the first to explore recombinant viruses as vaccine vectors. They used vaccinia virus, a highly immunogenic virus related to smallpox that is relatively nonvirulent in immunocompetent individuals. In most cases, a single immunization with recombinant vaccinia carrying a gene expressing an antigen will generate significantly greater immune responses against that antigen than the corresponding protein or peptide epitopes mixed with standard adjuvants.[211-213] This is particularly true for CTL generation. To date, many viruses have been explored as recombinant vaccine vectors, including attenuated replication-deficient poxviruses (such as modified vaccinia ancara,

fowlpox, and canarypox), adenovirus, herpesviruses, and Venezuelan equine encephalitis virus.[214-217] Each of these viruses has various advantages and disadvantages, and no clear "winner" has emerged as the absolute vector of choice. Features of viruses that can enhance their potency as vaccine vectors include their ability to induce immunologic "danger" signals at sites of infection and to directly infect APCs. Features of viruses that can diminish their potency as vaccine vectors include the presence of virally encoded inhibitors of immunity. These include molecules that block processing and presentation in the MHC class I pathway (such as TAP inhibitors and inhibitors of MHC class I traffic out of the endoplasmic reticulum) and cytokine decoys, to mention a few.[218] Deleting immunologic inhibitory genes from recombinant viruses may further enhance their vaccine potency while attenuating their virulence.

A major barrier to virus-based vaccination are neutralizing antibodies in preexposed or prevaccinated individuals that inhibit the initial round of infection and replication, thereby quenching their ability to immunize. Individuals who have never been previously exposed to the vaccinating virus generate neutralizing antibody after the first vaccination, thereby precluding subsequent vaccination with the same vector. This finding has led to the concept of cycling different viral vectors in "prime-boost" formats. Dramatic enhancement of immunization potency has been observed in prime-boost formats between both different viruses such as vaccinia followed by fowlpox between DNA vaccines and recombinant viral vaccines.[219,220]

Among the large number of clinical trials with viral vaccine vectors, the most extensive have involved poxvirus vectors. Based on enthusiasm from preclinical experiments, a number of prime-boost studies have been performed using vaccinia followed by fowlpox.[221] A Phase III trial in patients with inoperable pancreatic cancer was performed using a vaccinia-fowlpox prime-boost schedule versus chemotherapy or supportive care. The viral vectors incorporated two antigens—carcinoembryonic antigen and MUC-1—and also included ICAM-1, LFA-3, and B7.1 genes to putatively enhance the co-stimulatory activity of infected DCs (though this has never been proven). The trial was negative. Phase II trials with a similar prime-boost regimen for advanced prostate cancer using prostate-specific antigen (PSA) as the antigen have provided interesting results, but clinical benefit has not been definitively demonstrated. Regulatory hurdles and the inability to vaccinate repetitively are likely to preclude further development efforts for viral vaccines in cancer.

Engineered Bacteria

Genetic engineering of intracellular bacteria such as BCG, *Salmonella*, *Shigella*, and *Listeria* has produced a number of interesting and promising vaccines.[222-224] In principle, bacteria that enter antigen-presenting cells may represent a good vehicle for delivery of recombinant antigens. In certain cases, such as *Listeria*, the bacteria exhibit complex life cycles that involve both phagolysosomal and cytoplasmic stages. Thus, recombinant *Listeria monocytogenes* (LM) engineered to secrete antigens will load the MHC class II processing pathway during the phagolysosomal phase and the MHC class I pathway during the cytosolic phase of the life cycle. In addition, a number of recombinant bacteria actively induce infected APCs to secrete proinflammatory cytokines such as IL-12. More recently, recombinant bacteria have been used as vectors for delivery of DNA vaccines.[223,224] Thus, bacterial vaccines containing plasmids with eukaryotic promoter and enhancer elements driving the antigen gene result in potent immunization. These results indicate that the bacteria can directly transfer plasmids into eukaryotic transcriptional compartments within infected APCs.

The LM vectors are among the most promising bacterial vectors being developed for therapeutic vaccination of cancer. Dubensky and colleagues have identified a number of approaches to dramatically attenuate the virulence of LM without diminishing its immunogenicity. One approach is to knock out the ActA and Internalin(Inl)B genes of LM.[222] Knockout of ActA does not prevent the initial infection of cells with LM, but eliminates the capacity for cell-to-cell spread necessary for the propagation of LM infections. Knockout of the InlB gene eliminates the capacity of LM to infect hepatocytes while not affecting the capacity of LM to infect APCs. Thus, infection with InlB mutant LM generates strong intrahepatic inflammatory responses with minimal destruction of hepatocytes. ActA/InlB double mutant LM are equivalently immunogenic to wild-type LM, but are 4 to 5 logs attenuated in their virulence. Another approach to virulence attenuation of LM (which is applicable to other bacterial vectors as well) involves the knockout of DNA repair genes together with limited DNA crosslinking using psoralen derivatives. Because the DNA repair system has been knocked out, bacteria can be inhibited from replicating themselves with as little as a single DNA crosslink per bacterial genome. This approach maintains metabolic activity while formally "killing" the bacteria. These killed but metabolically active (KBMA) organisms maintain significant immunogenicity but have highly attenuated virulence.[225]

Despite the diversity of vaccine platforms just described, anecdotal reports of vaccine-induced tumor regressions, and promising Phase I and II clinical trial results with cancer vaccines evaluated since the 1960s, a string of failures in randomized clinical trials bred significant skepticism as to the ultimate clinical value of therapeutic cancer vaccines.[170] However, in the past couple of years, a number of important clinical successes with cancer vaccines have dramatically altered the perception of their potential value.

The first successful randomized Phase III cancer vaccine trial used a putative DC vaccine, sipuleucel-T, to treat patients with advanced hormone-resistant prostate cancer.[226] This vaccine is based on the concept that optimal T-cell activation requires antigen processing and presentation by a specialized cell—the DC—with the capacity to concomitantly deliver strong co-stimulatory signals in the form of membrane ligands and secreted cytokines. Sipuleucel-T is a patient-specific vaccine produced by transiently incubating the patient's own peripheral blood mononuclear cells (PBMCs) with a fusion protein consisting of PAP (prostatic acid phosphatase—a prostate/prostate cancer–specific antigen) linked to the DC growth and differentiation factor GM-CSF. A 4-month overall survival (OS) benefit relative to the control arm (uncultured PBMCs without PAP-GM-CSF fusion protein) in the absence of objective tumor regressions or effect on time to progression emphasizes a developing paradigm: that immunotherapy can potentially provide OS benefits that are not reflected in progression-free survival (PFS) or objective response rate (ORR). The survival benefit of sipuleucel-T ultimately led to FDA approval in 2010.

Recently, three positive randomized cancer vaccine trials have been reported. First, a trial comparing a poxvirus-PSA prime/boost vaccine regimen plus GM-CSF versus non–antigen-expressing viruses in patients with advanced prostate cancer demonstrated a significant (8 month) OS benefit for the vaccine arm but no effect on PFS or ORR.[227] Second, a melanoma vaccine consisting of a modified gp100 peptide plus systemic IL-2 was compared to systemic IL-2 alone in patients with advanced melanoma,[228] yielding a statistically higher ORR in the vaccine + IL-2 arm, improved PFS, and improved OS ($P = .06$). Of note, the same peptide vaccine, when combined with anti-CTLA-4, demonstrated no improvement in patients with advanced melanomas relative to anti-CTLA-4 alone (see later discussion), underscoring the importance of context when evaluating vaccines as components of combinatorial therapies. In 2013, a randomized trial comparing a GVAX/*Listeria*-mesothelin prime-boost vaccine versus GVAX alone in patients with advanced chemotherapy refractory pancreas cancer demonstrated a strong statistical survival benefit for the GVAX/*Listeria*-mesothelin prime-boost arm (6.1 versus 3.9 months, HR = 0.55, $P = .013$; per-protocol analysis: 9.7 versus 4.6 months, HR = 0.44, $P = .01$).[229] This prime-boost strategy was based on preclinical data demonstrating that priming with GVAX and boosting with a *Listeria* vaccine, but not vice versa, produced strongly enhanced T-cell responses and antitumor responses in animal models. The choice of mesothelin as antigen to incorporate into the *Listeria* (ActA/Internalin B KO as described earlier) vaccine vector was based on earlier demonstrations that it is highly upregulated in all pancreatic cancer, provides an advantage to tumor growth (and thus cannot be easily down-modulated without cost to the tumor cells), is expressed at low levels in a limited subset of mesothelial cells, and can be a clear target for induced T-cell responses.

In addition to therapeutic vaccine trials for established cancer, premalignant lesions caused by chronic viral infection are excellent targets for therapeutic vaccines. The best example of such a situation is HPV-associated premalignant lesions of the cervix, vulva, anus, and orolaryngeal mucosa. In a single-arm clinical trial using long peptides (which are selectively processed and presented by DCs) derived from HPV-16 E6 and E7 antigens induced complete regressions in 9 of 19 patients with vulvar intraepithelial neoplasia, an HPV-associated preneoplastic condition with a spontaneous regression rate of less than 2%.[230]

Co-inhibitory Ligands and Receptors That Down-Modulate Tumor Immunity

Without question, the major molecules to be successfully targeted in clinical cancer immunotherapy are the growing class of ligand-receptor pairs commonly referred to as immune checkpoints. In considering the mechanism(s) of action of inhibitors of various checkpoints, it is critical to appreciate the diversity of immune functions that they regulate. For example, the two immune checkpoint receptors that have been most actively studied in the context of clinical cancer immunotherapy, CTLA-4 (CD152) and PD-1 (CD279), regulate immune responses at very different levels and by very different mechanisms. The clinical activity of blocking antibodies for each of these receptors implies that antitumor immunity can be enhanced at multiple levels and that combinatorial strategies can be intelligently designed, guided by mechanistic considerations and preclinical models. This section focuses particular attention on the CTLA-4 and PD-1 pathways because they were the two checkpoints whose inhibition has revolutionized clinical cancer immunotherapy. However, it is important to emphasize that multiple additional checkpoints represent promising targets for therapeutic blockade based on preclinical experiments, and inhibitors of many of these are under active development.

The CTLA-4 Checkpoint—a Global Regulator of T-Cell Activation

CTLA-4, the first immune checkpoint receptor to be clinically targeted, is expressed exclusively on T cells, where it primarily regulates the amplitude of the early stages of T-cell activation. CTLA-4 knockout mice die within 3 weeks from immune destruction of multiple organs, which attests to its critical role as an inhibitory regulator of T-cell–dependent immune responses. Primarily, CTLA-4 counteracts the

activity of the T-cell co-stimulatory receptor CD28.[231-233] CD28 does not affect T-cell activation unless the TCR is first engaged by cognate antigen. Once antigen recognition occurs, CD28 signaling strongly amplifies the TCR signal to activate T cells. CD28 and CTLA-4 share identical ligands—CD80 (B7.1) and CD86 (B7.2).[234-238] Because CTLA-4 has a much higher overall affinity for both ligands, its expression on the surface of T cells dampens the activation of T cells by outcompeting CD28 in binding CD80 and CD86, as well as by actively delivering inhibitory signals to the T cell.[239-244] The specific signaling pathways by which CTLA-4 blocks T-cell activation are still under investigation, although a number of studies suggest that activation of the phosphatases SHP2 and PP2A is important in counteracting kinase signals induced by TCR and CD28.[232] However, CTLA-4 also confers "signaling independent" T-cell inhibition through sequestration of CD80 and CD86 from CD28 engagement as well as active removal from the APC surface.[245] The central role of CTLA-4 in maintaining T-cell activation in check is dramatically demonstrated by the systemic immune hyperactivation phenotype of CTLA-4 knockout mice.[246,247]

Even though CTLA-4 is expressed by activated CD8 killer T cells, the major physiologic role of CTLA-4 appears to be through distinct effects on the two major subsets of CD4 T cells—down-modulation of helper T-cell activity and enhancement of regulatory T-cell suppressive activity.[231,248,249] CTLA-4 blockade results in a broad enhancement of immune responses dependent on helper T cells, and conversely, CTLA-4 engagement on Treg (Tregs) enhances their suppressive function. CTLA-4 is a target gene of the transcription factor Foxp3,[77,250] the expression of which determines the Treg lineage,[251,252] and Tregs therefore express CTLA-4 constitutively. Although the mechanism by which CTLA-4 enhances the inhibitory function of Tregs is not known, Treg-specific CTLA-4 knockout or blockade significantly inhibits their ability to regulate both autoimmunity and antitumor immunity.[248,249] Thus, in considering the mechanism of action for CTLA-4 blockade, both enhancement of effector CD4 T-cell activity and inhibition of Treg-dependent immune suppression are likely important factors.

Clinical Application of CTLA-4 Blocking

Blockade of CTLA-4 as a general strategy was initially questioned because there is no tumor specificity to expression of the CTLA-4 ligands (other than certain myeloid and lymphoid tumors) and also because the dramatic lethal autoimmune/hyperimmune phenotype of CTLA-4 knockout mice predicted a high degree of immune toxicity associated with blockade of this receptor. However, Allison and colleagues used preclinical models to demonstrate that a therapeutic window was indeed achieved when CTLA-4 was partially blocked with antibodies.[253] The initial studies demonstrated significant antitumor responses without overt immune toxicities when mice bearing partially immunogenic tumors, particularly melanomas, were treated with anti-CTLA-4 antibodies as single agents. Poorly immunogenic tumors did not respond to anti-CTLA-4 as a single agent but did respond when anti-CTLA-4 was combined with a GM-CSF–transduced cellular vaccine.[254] These findings suggested that, if there was an endogenous antitumor response present in the animals after tumor implantation, CTLA-4 blockade could enhance that endogenous response, which ultimately induced tumor regression. In the case of poorly immunogenic tumors, which do not induce significant endogenous responses, the combination of a vaccine and an anti-CTLA-4 antibody could induce a strong enough immune response to slow tumor growth and in some cases eliminate established tumors.

These preclinical findings encouraged the production and testing of two fully human anti-CTLA-4 antibodies, ipilimumab and tremelimumab, which began clinical testing in 2000. As with virtually all anticancer agents, initial testing was as a single agent in patients with advanced disease, not responding to conventional therapy.[255] Both antibodies produced objective clinical responses in roughly 10% of melanoma patients but also immune-related toxicities involving various tissue sites in 25% to 30% of patients, with colitis being a particularly common event.[256-258] The first randomized Phase III clinical trial to be completed was for tremelimumab in patients with advanced melanoma. In the trial, 15 mg/kg tremelimumab was given every 3 months as a single agent and compared to DTIC, a standard melanoma chemotherapy treatment. The trial showed no survival benefit with this dose and schedule relative to DTIC.[259]

However, ipilimumab fared better. Even though the two antibodies appear to have similar intrinsic activity, response rates in Phase II trials, and immune toxicity profiles, ipilimumab was more carefully evaluated at different doses and schedules. In addition, more careful definition of algorithms for improved clinical management of the immune toxicities (using steroids and TNF blockers) mitigated the overall morbidity and mortality associated with immunologic toxicities.

The toxicity rate for ipilimumab is quite significant (14% to 30% grade 3-5 in various studies) and is generally immunologic in nature, implying that it is "on target." This was predicted from the dramatic lethal hyperimmune/autoimmune phenotype of the CTLA-4 KO mice. The most common toxicities with both ipilimumab and tremelimumab are cutaneous (rash) and colitis. However, hepatitis, pneumonitis, hypophysitis, and thyroiditis are also observed.

Interestingly, although there is evidence that clinical responses might be associated with immune-related adverse events, this correlation is modest.[260]

Finally, in a randomized three-arm clinical trial of patients with advanced melanoma who received a melanoma-specific gp100 peptide vaccine alone, the gp100 vaccine plus ipilimumab, or ipilimumab alone, there was a 3.5-month survival benefit for patients in both groups receiving ipilimumab (i.e., with or without the gp100 peptide vaccine) compared with the group receiving peptide vaccine alone.[261] As the first therapy ever to demonstrate a survival benefit for patients with metastatic melanoma (DTIC was approved based on response rate and has never been shown to provide a survival benefit in melanoma), ipilimumab was FDA approved for the treatment of advanced melanoma in 2010.

More impressive than the mean survival benefit was the affect on long-term survival: 20% of the ipilimumab-treated patients survived beyond 2 years (compared with 5% of patients receiving the peptide vaccine alone).[261] In this and other studies, the proportion of long-term survivors is higher than the proportion of objective responders. The finding of ongoing responses and survival long after the completion of a relatively short course of therapy (four doses of 10 mg/kg over 3 months) supports the concept that immune-based therapies might reeducate the immune system to maintain tumors in check after completion of the therapeutic intervention.

As with all oncology agents that benefit a limited proportion of treated patients, much effort has been expended to define biomarkers predictive of clinical response to anti-CTLA-4 treatment. To date, no such pretreatment biomarker has been validated to the point where it could be applied as part of standard-of-care therapeutic decision making, though insights have emerged from the identification of certain posttreatment immune responses that seem to correlate with clinical outcome.[262-264]

An important feature of the anti-CTLA-4 clinical responses that distinguishes them from conventional chemotherapeutic agents and oncogene-targeted small-molecule drugs is their kinetics. Whereas chemotherapy and tyrosine kinase inhibitor (TKI) responses commonly occur within weeks of initial administration, the response to immune checkpoint blockers is slower and, in a number of patients, delayed (up to 6 months after treatment initiation). In some cases, metastatic lesions actually increase on computed tomography or magnetic resonance imaging scans before regressing. These findings demand a reevaluation of response criteria for immunotherapeutics that does not use conventional time-to-progression or RECIST objective response criteria, which were developed based on experience with chemotherapy agents and as the primary measure of drug efficacy.[265]

Biology of the PD-1 Checkpoint—a Pathway That Functions within the Tumor Microenvironment

Another immune checkpoint receptor, PD-1, is emerging as a promising target, emphasizing the diversity of potential molecularly defined immune manipulations capable of inducing antitumor responses by the patient's own immune system.

In contrast to CTLA-4, the major role of PD-1 is to limit the activity of T cells in the peripheral tissues at the time of an inflammatory response to infection and to limit autoimmunity.[266-272] This translates to a major immune resistance mechanism within the tumor microenvironment (Figure 52-4).[273-275] PD-1 expression is induced when T cells become activated.[267] When engaged by one of its ligands, PD-1 inhibits kinases involved in T-cell activation via the phosphatase SHP2,[266] although additional signaling pathways are also likely induced, and because PD-1 engagement inhibits the TCR stop signal, this pathway could modify the duration of T-cell/APC or T-cell/target cell contact.[276] Similar to CTLA-4, PD-1 is highly expressed on Tregs, where it may enhance their proliferation in the presence of ligand.[277] Because many tumors are highly infiltrated with Tregs that likely further suppress effector responses, PD-1 pathway blockade may also enhance antitumor responses by diminishing the number and/or suppressive activity of intratumoral Tregs.

The two ligands for PD-1 are PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273).[266,278-280] These B7 family members share 37% sequence homology and arose via gene duplication, positioning them within 100 kb of each other in the genome.[280] Recently, an unexpected molecular interaction between PD-L1 and CD80 was discovered,[281] whereby CD80 expressed on T cells (and possibly APCs) can potentially behave as a receptor rather than a ligand, delivering inhibitory signals when engaged by B7-H1.[282,283] The relevance of this interaction in tumor immune resistance has not yet been determined. Finally, genetic evidence from PD-1–deficient T cells suggests that both PD-L1 and may bind to a co-stimulatory receptor expressed on T cells.[282] These complex binding interactions are reminiscent of the CD80/CD86 ligand pair, which binds the co-stimulatory CD28 expressed on resting T cells and the inhibitory CTLA-4 expressed on activated T cells, though, as stated earlier, PD-1 predominantly regulates effector T-cell activity within tissue and tumors, whereas CTLA-4 predominantly regulates T-cell activation. Understanding the role of these various interactions in given cancer settings is highly relevant for the selection of both antibodies and recombinant ligands for use in the clinic.

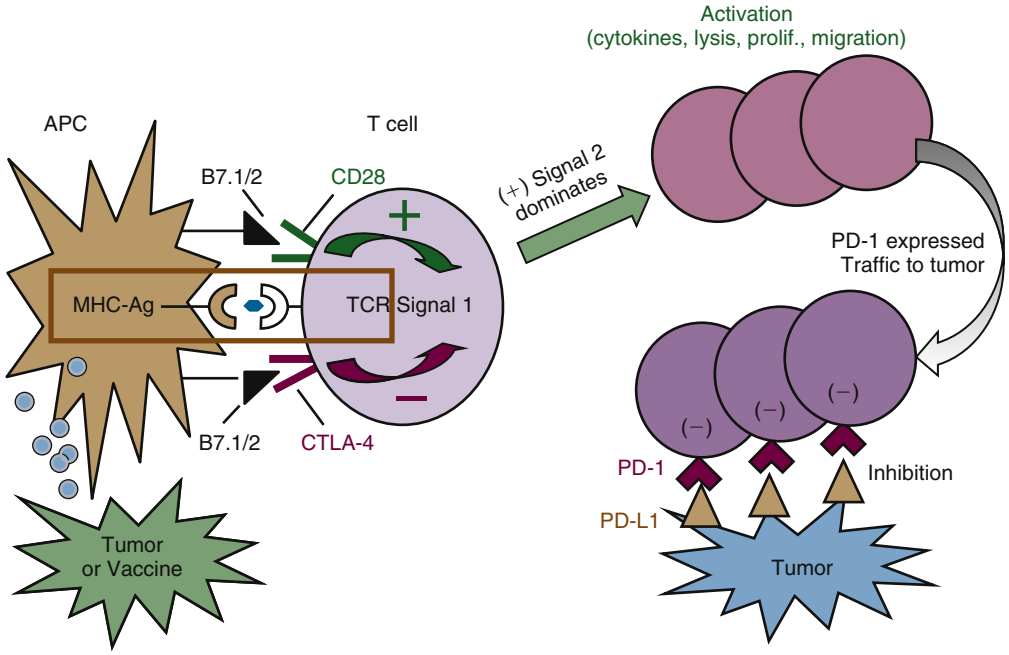


Figure 52-4 CTLA-4 and PD-1 checkpoints act to regulate different elements of the T-cell response to tumors Naïve T cells and resting T cells express little CTLA-4 or PD-1 on their surface. On initial T-cell activation via triggering of the T-cell receptor (TCR) by cognate peptide/MHC complexes together with engagement of CD28 by B7-1 and/or B7-2, CTLA-4 becomes expressed on the cell surface while the T cell is still engaged with its antigen-presenting cell (generally a dendritic cell), usually in the secondary lymphoid tissue. The greater the TCR stimulus, the more CTLA-4 is expressed on the surface. Inhibitory signals from CTLA-4 interaction of CTLA-4 with B7-1 and B7-2 results in a counterregulatory signal that down-modulates the ultimate amplitude of T-cell activation. Activated T cells then traffic into the tumor, where the PD-1 pathway becomes important. The PD-1 checkpoint primarily operates within the tumor where PD-1 ligands (PD-L1 and sometimes PD-L2) are upregulated.

PD-1 is more broadly expressed than CTLA-4; it is induced on other activated non–T lymphocyte subsets, including B cells and NK cells,[284,285] limiting their lytic activity. Thus, although PD-1 blockade is typically viewed as enhancing the activity of effector T cells in tissues and in the tumor microenvironment, it likely also enhances NK activity in tumors and tissues and may also enhance antibody production either indirectly or through direct effects on PD-1$^+$ B cells.[286]

In addition, chronic antigen exposure, such as occurs with chronic viral infection and cancer, can lead to high levels of persistent PD-1 expression, which induces a state of exhaustion or anergy among cognate antigen-specific T cells. This state, which has been demonstrated in multiple murine and human chronic viral infections, appears to be partially reversible by PD-1 pathway blockade.[287] Finally, although the PD-1 pathway plays its major role in limiting immune effector responses in tissues (and tumors), it can also shift the balance from T-cell activation to tolerance at early stages in T-cell responses to antigen within secondary lymphoid tissues (i.e., at a similar point compared to CTLA-4). Taken together, these findings imply a complex set of mechanisms of action for PD-1 pathway blockade.

PD-1 is expressed on a large proportion of tumor-infiltrating lymphocytes (TILs) from many different tumor types.[288,289] Some of the enhanced PD-1 expression among CD4 TILs reflects a generally high level of PD-1 on Tregs, which, as noted earlier, can represent a large fraction of intratumoral CD4 T cells. Increased PD-1 expression on CD8 TILs may reflect an anergic/exhausted state, as has been suggested by decreased cytokine production by PD-1$^+$ versus PD-1$^-$ TILs from melanomas.[288]

Just as PD-1 is highly expressed on TILs from many cancers, the PD-1 ligands are commonly upregulated on many different human tumors.[274,290] On solid tumors, the major PD-1 ligand to be expressed is PD-L1. Forced expression of PD-L1 on murine tumors inhibits local antitumor T-cell responses.[274,291] Indeed, this combination of findings provides the basis for PD-1 pathway blockade to enhance antitumor effector function in the tumor microenvironment. As immunohistochemistry (IHC) techniques and flow cytometry analysis of surface expression have been employed, it has become clear that the selective upregulation of PD-1 ligands in various human tumor types is heterogeneous at a number of levels.[275] Expression patterns of PD-1 ligands may very well be critical in choosing suitability for therapeutic blockade of this pathway, because its primary role in cancer is thought to be immune inhibition within the tumor microenvironment, and PD-1 inhibits lymphocyte function only when it is engaged by cognate ligand.

Initially, the majority of melanoma, ovarian, and lung cancer samples were reported to have high expression of PD-L1,[274,291,292] and subsequently, many other human

cancers were reported to upregulate PD-L1. In addition to tumor cells, PD-L1 is commonly expressed on myeloid cells in the tumor microenvironment.[293-295] An initial report in renal cancer demonstrated that expression of PD-L1 on either tumor cells or infiltrating leukocytes in primary tumors predicted a worse prognosis—that is, decreased overall survival relative to PD-L1$^-$ tumors.[296] Since that report, analyses of various tumors have suggested that PD-L1 status can correlate with poor prognosis or better prognosis or can show no correlation with prognosis.[275,297-301] Variability in IHC technique, cancer type, stage of cancer analyzed (most analyses are of primary, not metastatic lesions), and treatment history in the analyzed cohort all likely contribute to the wide range of reported outcomes.

Although most analyses of PD-1 ligand expression have focused on PD-L1, PD-1 has also been reported to be upregulated on a number of tumors. It is highly upregulated on certain B-cell lymphomas such as primary mediastinal, follicular cell B-cell lymphoma, and Hodgkin's disease.[299] Upregulation in these lymphomas is commonly associated with gene amplification or rearrangement to the CIITA locus, which is highly transcriptionally active in B-cell lymphomas.[302]

Given the heterogeneity of expression and potential relevance as a biomarker for blockade of the PD-1 pathway, it is important to understand the signals that induce expression of PD-1 ligands on tumor cells and also hematopoietic cells within the tumor microenvironment (Figure 52-5). Two general mechanisms for the regulation of PD-L1 have emerged: innate and adaptive. For some tumors such as glioblastoma, it has been demonstrated that PD-L1 is driven by constitutive oncogenic signaling pathways in the tumor cell. Expression on glioblastomas is enhanced on deletion or silencing of PTEN, implicating the PI3K-AKT pathway.[303] Similarly, constitutive ALK signaling, observed in certain lymphomas and occasionally in lung cancer, has been reported to drive PD-L1 expression via STAT3 signaling.[304]

The alternative mechanism for PD-L1 upregulation on tumors that has emerged from both clinical and preclinical studies reflects their adaptation to endogenous tumor-specific immune responses—a process termed *adaptive resistance*.[275] In adaptive resistance, the tumor uses the natural physiology of the PD-1 ligand induction for tissue protection in the face of an immune response to infection in order to protect itself from an antitumor response. Expression of PD-L1 as an adaptive response to endogenous antitumor immunity can occur because it is induced on most cancers in response to interferons—predominantly γ-interferon, similarly to epithelial and stromal cells in normal tissues.[305-307] This mechanism represents an alternative to the conventional drug resistance mechanisms that involve mutation of drug targets. It also contrasts with mechanisms of viral immune escape that involve mutation of immunodominant epitopes. The mechanism of adaptive resistance intrinsically implies that immune surveillance does exist even in some advanced cancers, but the tumor ultimately resists immune elimination by upregulating ligands for inhibitory receptors on tumor-specific lymphocytes that turn off antitumor responses within the tumor microenvironment.

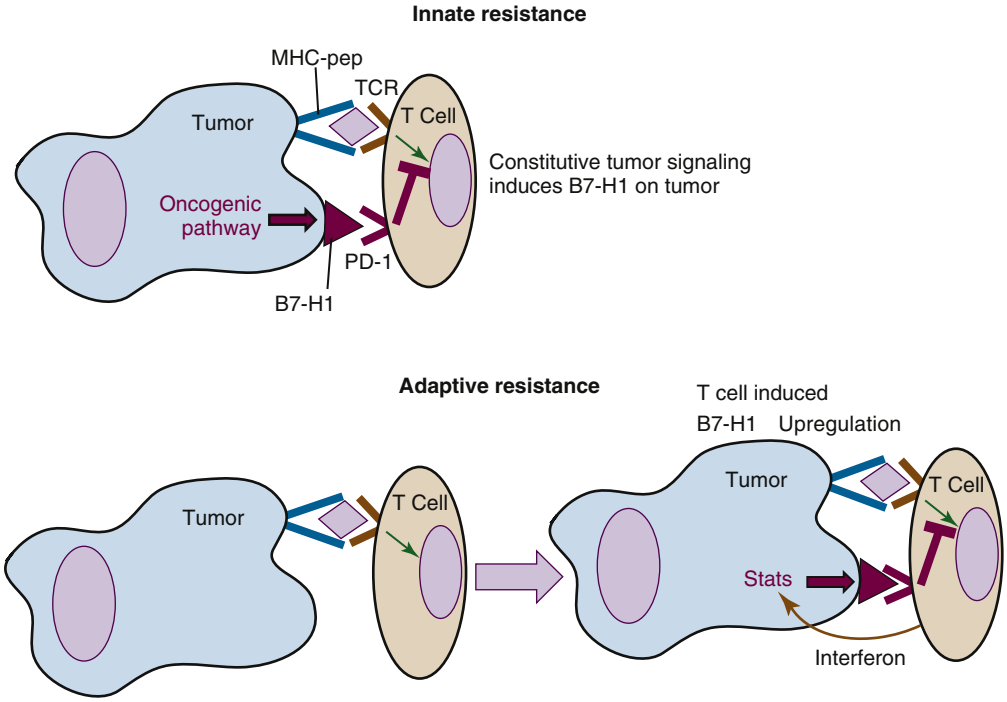


Figure 52-5 Two mechanisms for PD-L1 induction on tumors: innate and adaptive PD-L1 can be constitutively expressed on tumors as a consequence of oncogene-driven transcriptional activation. Alternatively, PD-L1 can be induced in an adaptive fashion when there are the right inflammatory cytokines in the tumor microenvironment consequent to an ongoing immune response to the tumor. This mechanism of tumor resistance to immune attack is coopted from physiologic PD-L1 expression for tissue protection in the setting of antimicrobial immune responses. Innate and adaptive mechanisms of PD-L1 expression on tumors can coexist. The adaptive resistance mechanism implies that PD-L1 expression is a "marker" of endogenous antitumor immunity.

A number of preclinical and clinical studies support the adaptive resistance hypothesis. Gajewski and colleagues have demonstrated that melanomas can be roughly divided into "inflammatory" and "noninflammatory" categories defined by expression of multiple inflammatory genes, including those involved in the interferon pathway.[308] A recent study in melanoma demonstrated a very high correlation between cell surface PD-L1 expression on tumor cells and both lymphocytic infiltration and intratumoral γ-IFN expression. This correlation was seen not only among tumors but within individual PD-L1$^+$ tumors at the regional level, in which regions of lymphocyte infiltration were exactly the regions where PD-L1 was expressed on both tumor cells and infiltrating leukocytes.[275]

Evidence of Clinical Activity for PD-1 Blockade

Taken together, the general findings of increased PD-1 expression by TIL and increased PD-1 ligand expression by tumor cells created an important rationale for the capacity of antibody blockade of this pathway to enhance intratumoral immune responses. This was validated through many murine tumor studies demonstrating enhanced antitumor immunity through antibody blockade of PD-1 or its ligands (see earlier discussion). Furthermore, the relatively mild phenotypes of PD-1, PD-L1, and PD-L2 knockout mice suggest that blockade of this pathway would result in less collateral immune toxicity than CTLA-4 blockade, a finding that appears to be the case in clinical trials.

Although the clinical experience with anti-PD-1 antibodies is less extensive than with anti-CTLA antibodies at this time, results look extremely promising. In the first Phase I clinical trial with a fully human IgG4 anti-PD-1 antibody, there were a number of cases of tumor regression, including mixed responses, partial responses, and a complete response.[309] Tumor regressions were observed in four of the five histologies examined—melanoma and colon, renal, and lung cancer—and were associated with significant increases in lymphocyte infiltration into metastatic tumor deposits. Results from a second larger clinical trial, sponsored by BMS and extending the treatment with anti-PD-1 (named nivolumab) to 2 years, demonstrated objective responses in 31% of patients with advanced melanoma, with an additional 7% achieving disease stabilization for more than 6 months. Similar response rates were observed in renal cancer with an additional 27% with disease stabilization for more than 6 months. Most surprisingly, there was an 18% response rate in non–small-cell lung cancer (NSCLC), with an additional 7% disease stabilization for longer than 6 months. Efficacy against melanoma, renal cancer, and lung cancer was also observed with an anti-PD-L1 antibody.[310]

Among 270 nivolumab-treated patients with lung, melanoma, or kidney cancer, 1- and 2-year landmark survival rates were 42%/14% for lung cancer, 62%/43% for melanoma, and 70%/50% for kidney cancer. Median overall survival in these heavily pretreated patients (47% with three to five prior systemic therapies) was 9.6, 16.8, and more than 22 months, respectively. Among all responders, median response duration was 74, 104, and 56 weeks, respectively. Among 19 responders who discontinued therapy for reasons other than disease and were followed for at least 4 months (range 4 to 14 months), 70% retained their response.[311]

As predicted by the distinct phenotypes of the PD-1 knockout versus CTLA-4 knockout mice, the frequency of immune-related toxicities from anti-PD-1 treatment appears to be less than from anti-CTLA-4. Grade 3/4 drug-related toxicity was less than 15% and was also largely immune related. In contrast to anti-CTLA-4, the most significant toxicity was pneumonitis, which produced a 1% mortality rate. Recently instituted protocols to manage pneumonitis with steroids and, when necessary, anti-TNF blocking antibodies appear to mitigate lung toxicity.

It is logical to imagine that the enhancement of antitumor immune responses on blockade of this pathway would depend in significant part on expression of a ligand for PD-1 within the tumor. Analysis of 42 patients treated with anti-PD-1 in the trial described earlier demonstrated a strong correlation between PD-L1 expression and response. None of 17 patients with no membrane PD-L1 expression on pretreatment biopsies responded to anti-PD-1, whereas 44% patients with more than 5% of tumor cells expressing membrane PD-L1 displayed either an objective or mixed response.[312] The lack of response in patients whose tumors exclusively expressed cytosolic PD-L1 was also notable, as cytosolic PD-L1 would fail to activate the PD-1 pathway. If validated in a larger series, this finding sets the stage for a broader assessment of immune checkpoint ligands and receptors as targets for antibody blockade as well as an assessment of ligand expression in the tumor as a biomarker for success in blockade of a specific checkpoint pathway.

There are a number of companies developing and testing antibodies that block the PD-1 pathway; a recent study with a different anti-PD-1 antibody produced by Merck (named lambrolizumab) demonstrated a 38% response rate in melanoma,[313] and an anti-PD-L1 antibody produced by Genentech gave similar response rates in melanoma and NSCLC (but a somewhat lower response rate in kidney cancer) to the BMS anti-PD-1 antibody.[314] These results validate the PD-1 pathway as an important target for immunotherapeutic targeting. Based on the known interactions between the PD-1 ligands, it is theoretically possible that a

PD-1 antibody would have distinct biologic activity from an anti-PD-L1 antibody—an anti-PD-1 antibody would block PD-1 interaction with both PD-L1 and PD-L2 but not the interaction between PD-L1 and CD80. Most anti-PD-L1 antibodies block the interaction between PD-L1 and CD80 and between PD-L1 and PD-1 but would not block PD-1 interaction with PD-L2. Thus, it is possible that, depending on which interactions dominate in a particular cancer, PD-1 and PD-L1 antibodies might not have redundant activity.

Based on the distinct roles of CTLA-4 and PD-1 in regulating distinct components of the immune response, it was postulated that combined blockade of these pathways might provide an additive or synergistic antitumor effect. Indeed, a recent study demonstrated a 41% response rate in melanoma patients treated concurrently with ipilimumab and nivolumab. A larger proportion of the responses were "deep" (more than 80%) than observed with nivolumab alone, and there were about 20% additional mixed responses and stable disease longer than 6 months. However, toxicity was also greater than with nivolumab alone, with a 53% grade 3/4 toxicity rate.[315] Although the ultimate long-term clinical benefit of this combination remains to be determined, the study emphasizes the potential for combinatorial blockade of multiple checkpoints.

Additional Checkpoints Participate in Tumor Immune Resistance and Tolerance

Successful clinical outcomes of CTLA-4 and PD-1 pathway targeting have garnered great interest in a number of additional checkpoints (Figure 52-6). Basic immunologic studies have demonstrated that a number of checkpoint receptors are expressed coordinately under circumstances of tolerance to self-antigens and chronic infections as well as in inflammatory settings. In addition to defined lymphocyte inhibitory receptors, a number of B7-family inhibitory ligands—in particular B7-H3 (CD276) and B7-H4—do not yet have defined receptors, but murine knockout experiments support an inhibitory role for both these molecules.[316] In addition, they are upregulated on tumor cells or tumor-infiltrating cells.[317] B7-H3 appears to be upregulated on endothelial cells of the tumor vasculature, and B7-H4 has been reported to be expressed on tumor-associated macrophages.[316] Preclinical tumor models have been used to demonstrate that blockade of many of these individual immune checkpoint ligands or receptors can enhance antitumor immunity and dual blockade of coordinately expressed receptors can produce additive or synergistic antitumor activity. Inhibitors for a number of these immune checkpoint targets either are entering the clinic or are under active development. Those described next are targets with currently available blocking antibodies or small-molecule inhibitors but do not represent a comprehensive list.

LAG-3 (CD223), 2B4 (CD244), BTLA (CD272), Tim-3, A2aR, and the family of killer inhibitory receptors have each been associated with inhibition of lymphocyte activity and in some cases induction of lymphocyte anergy. Antibody targeting of these receptors, either alone or in combination with a second immune checkpoint blocker, has been shown to enhance antitumor immunity in animal models of cancer. Because many tumors express multiple inhibitory ligands and TILs express multiple inhibitory receptors, there are many opportunities to enhance antitumor immunity via dual or triple blockade of immune checkpoints. Although human blocking antibodies specific for a number

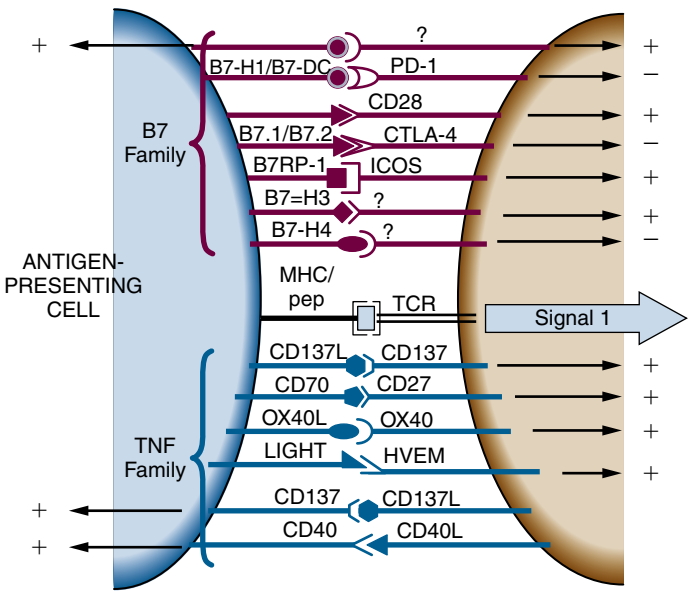


Figure 52-6 Multiple co-stimulatory and co-inhibitory ligand-receptor interactions ultimately determine the amplitude of T-cell activation and the potency of effector T-cell responses in tissue and tumor B7 family ligands and CD28 family receptors are shown in *purple* and TNF/TNFR family ligand-receptor pairs are shown in *blue*. There are additional inhibitory ligand-receptor pairs that do not fit into either of these families. Some of the receptors for B7 family members are not yet discovered. Whereas TNF/TNFR interactions are usually one-on-one pairs, B7 family ligands often interact with multiple receptors. HVEM is a TNFR family member—in addition to its interaction with the TNF member LIGHT, it also interacts with the inhibitory receptor BTLA, which is a member of the CD28 family. Additional signals of activation or inhibition are contributed by cytokines.

of these "second generation" inhibitory receptors are under development, none has entered the clinic at this time. Most of these receptors are induced on T-cell activation, in keeping with the biologic theme that they play roles in feedback inhibition of T-cell responses when their cognate ligands are present. In addition to providing inhibitory signals to activated effector T cells, some of these receptors such as LAG-3 are highly expressed on Tregs, where they are important to amplify their inhibitory activity.[79] This implies that, as with CTLA-4 and PD-1, these receptors play a dual role in ultimately inhibiting effector immune responses and blocking antibodies, and therefore have multiple potential mechanisms of action.

LAG-3 was cloned more than 20 years ago as a CD4 homologue,[318] but its function in the immune checkpoint was only defined in 2005, when it was shown to play a role in enhancing Treg function.[79,319] LAG-3 also inhibits CD8 effector function independently of its role on Tregs.[320] The only known ligand for LAG-3 is MHCII, which is upregulated on some epithelial cancers (generally in response to IFN-γ) but is also expressed on tumor-infiltrating macrophages and dendritic cells. The role of the LAG-3/MHCII interaction in LAG-3 mediated inhibition of T-cell responses is unclear because anti-LAG-3 antibodies that do not block the LAG-3/MHCII interaction nonetheless enhance T-cell proliferation and effector function in vitro and in vivo. The MHCII interaction of LAG-3 may be most important for its role in enhancing Treg function. LAG-3 is one of a number of immune checkpoint receptors coordinately upregulated on both Tregs and anergic T cells and simultaneous blockade can result in enhanced reversal of this anergic state relative to blockade of either receptor. In particular, PD-1 and LAG-3 are commonly coexpressed on anergic or exhausted T cells.[321,322] Dual blockade of LAG-3 and PD-1 provide synergy in reversing anergy among tumor-specific CD8 T cells as well as virus-specific CD8 T cells in the setting of chronic infection. Dramatic evidence of the effects of coordinate T-cell inhibition by PD-1 and LAG-3 comes from PD-1/LAG-3 double knockout mice, which completely reject even poorly immunogenic tumors in a T-cell–dependent fashion, but also develop autoimmune syndromes much more quickly than PD-1 or LAG-3 single knockouts that are ultimately fatal (though not as quickly as CTLA-4 knockouts).[323] These findings emphasize the balance between antitumor effects and autoimmune side effects that must be taken into consideration in all of the immune checkpoint blockade strategies.

Tim-3, the ligand of which is galectin-9 (a galectin reported to be upregulated in a number of cancer types such as breast cancer) inhibits Th1 responses,[324] and anti-Tim-3 antibodies enhance immunity.[325] Tim3 has also been reported to be coexpressed with PD-1 on tumor-specific CD8 T cells, and dual blockade of both molecules significantly enhances the in vitro proliferation and cytokine production of human T cells when stimulated by the cancer-testes antigen NY-ESO-1. In animal models, coordinate blockade of PD-1 and Tim3 was reported to enhance antitumor responses and tumor rejection under circumstances where only modest effects from blockade of each individual molecule were observed.[326-328]

BTLA was first identified as an inhibitory receptor on T cells based on enhanced T-cell responses observed in the BTLA knockout mice.[329] Subsequently, herpesvirus entry mediator (HVEM), which is expressed on certain tumor cell types (i.e., melanoma) as well as tumor-associated endothelial cells, was demonstrated to be the BTLA ligand.[330] This is a rare case in which a TNF family member interacts with an immunoglobulin supergene family member. BTLA expression on activated virus-specific CD8 T cells is relatively low, but it has been demonstrated to be much more highly expressed on TILs from melanoma patients. BTLA(hi) T cells are inhibited in the presence of its ligand, HVEM. Thus, BTLA may also be a relevant inhibitory receptor for T cells in the tumor microenvironment.[331] The system of HVEM-interacting molecules is complex—two additional interacting molecules, CD160 (an immunoglobulin superfamily member) and LIGHT (a TNF family member), appear to mediate inhibitory and co-stimulatory activity, respectively. It also appears that signaling can be bidirectional depending on the specific combination of interactions. The complexity of this system makes therapeutic inhibition strategies less straightforward than with other inhibitory receptors or ligands, although dual blockade of BTLA and PD-1 clearly enhances antitumor immunity.[332]

The A2a receptor for adenosine inhibits T-cell responses, in part by driving CD4 T cells to express Foxp3 and develop into Tregs.[333] Knockout of this receptor results in enhanced and sometimes pathologic inflammatory responses to infection. This receptor is particularly relevant in tumor immunity because the rate of cell death in tumors from cell turnover is high, and dying cells release adenosine. In addition, Tregs express high levels of the exoenzymes CD39, which converts extracellular ATP to AMP, and CD73, which converts AMP to adenosine.[334] Given that A2a receptor engagement by adenosine drives T cells to become Tregs, this can produce a self-amplifying loop within the tumor. Indeed, tumors grow more slowly in A2aR knockout mice, and tumor vaccines are much more effective against established tumors in these mice.[335] A2aR can be inhibited either by antibodies that block adenosine binding or by adenosine analogues, some of which are fairly specific for A2aR. Although these drugs have been used in clinical trials for Parkinson's disease, they have not yet been tested clinically in cancer patients.

Killer inhibitory receptors are a broad category of inhibitory receptors that can be divided into two classes based on structure: killer immunoglobulin receptors (KIR) and C-type lectin receptors, which are type II membrane receptors.[336-338] These receptors were originally described as critical regulators of the killing activity of NK cells, though many are expressed on T cells and APCs.[339] The importance of their inhibitory role on T cells and APCs (i.e., DCs) is less well studied, but the resulting activation of NK cells can provide potent antitumor activity. Many of the killer inhibitory receptors are specific for subsets of HLA molecules and possess allele specificity. However, other receptors recognize broadly expressed molecules—for example, the C-type lectin receptor KLRG1 recognizes e-cadherin. The potential value of NK cells in antitumor responses when their inhibitory receptors are not appropriately engaged is best exemplified by the significantly enhanced graft-versus-tumor effects in allogeneic bone marrow transplants elicited by mismatches between donor NK inhibitory receptors and recipient HLA alleles. The big question in therapeutic blockade of NK inhibitory receptors is which among the more than 20 receptors should be targeted.

Summary

After decades of research and clinical trials aimed at harnessing cancer patients' immune systems to attack their cancer, clinical efficacy with both vaccines and inhibitors of immune checkpoints has been demonstrated. The past few years have therefore become a turning point, establishing active immunotherapy as a viable approach to cancer therapy. These advances have been fueled by basic molecular and cellular discoveries related to immune system activation as well as study of the tumor microenvironment to identify resistance mechanisms that can be targeted directly. Future work will concentrate on targeting multiple pathways of immune regulation and developing rationally designed combinatorial approaches.

Key References

16. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
23. Sharkey MS, Lizee G, Gonzales MI, et al. CD4+ T-cell recognition of mutated B-RAF in melanoma patients harboring the V599E mutation. *Cancer Res*. 2004;64:1595-1599.
24. Somasundaram R, Swoboda R, Caputo L, et al. Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. *Cancer Res*. 2006;66:3287-3293.
25. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
26. Thomas AM, Santarsiero LM, Lutz ER, et al. Mesothelin-specific CD8+ T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med*. 2004;200:297-306.
30. Madsen B, Tarsounas M, Burchell JM, et al. PLU-1, a transcriptional repressor and putative testis-cancer antigen, has a specific expression and localisation pattern during meiosis. *Chromosoma*. 2003;112:124-132.
37. Lutz E, Yeo CJ, Lillemoe KD, et al. A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation. *Ann Surg*. 2011;253:328-335.
57. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6:836-848.
58. Fenner JE, Starr R, Cornish AL, et al. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat Immunol*. 2006;7:33-39.
61. DuPage M, Mazumdar C, Schmidt LM, et al. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature*. 2012;482:405-409.
62. Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature*. 2005;437:141-146.
63. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. 2007;450:903-907.
70. Drake CG, Doody AD, Mihalyo MA, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. *Cancer Cell*. 2005;7:239-249.
72. Deng L, Langley RJ, Brown PH, et al. Structural basis for the recognition of mutant self by a tumor-specific, MHC class II-restricted T cell receptor. *Nat Immunol*. 2007;8:398-408.
73. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27.
75. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.
77. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.
78. Bruder D, Probst-Kepper M, Westendorf AM, et al. Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol*. 2004;34:623-630.
79. Huang CT, Workman CJ, Flies D, et al. Role of LAG-3 in regulatory T cells. *Immunity*. 2004;21:503-513.
81. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*. 2007;8:277-284.
82. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol*. 2007;8:457-462.
86. Ercolini AM, Ladle BH, Manning EA, et al. Recruitment of latent pools of high-avidity CD8+ T cells to the antitumor immune response. *J Exp Med*. 2005;201:1591-1602.
88. Telang S, Rasku MA, Clem AL, et al. Phase II trial of the regulatory T cell-depleting agent, denileukin diftitox, in patients with unresectable stage IV melanoma. *BMC Cancer*. 2011;11:515.
89. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother*. 2006;55:237-245.
92. Bronte V, Serafini P, De Santo C, et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J Immunol*. 2003;170:270-278.
94. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65:3044-3048.
97. Baban B, Hansen AM, Chandler PR, et al. A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type I IFN signaling following B7 ligation. *Int Immunol*. 2005;17:909-919.
98. Mellor AL, Chandler P, Baban B, et al. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3-dioxygenase. *Int Immunol*. 2004;16:1391-1401.

99. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest*. 2004;114:280-290.
100. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med*. 2003;9:1269-1274.
101. Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*. 2011;478:197-203.
102. Mezrich JD, Fechner JH, Zhang X, et al. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol*. 2010;185:3190-3198.
103. Favre D, Mold J, Hunt PW, et al. Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med*. 2010;2:32ra36.
113. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A*. 2005;102:419-424.
125. Fritz JH, Ferrero RL, Philpott DJ, et al. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol*. 2006;7:1250-1257.
126. Honda K, Sakaguchi S, Nakajima C, et al. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc Natl Acad Sci U S A*. 2003;100:10872-10877.
127. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*. 2006;441:101-105.
141. Kelland L. Discontinued drugs in 2005: oncology drugs. *Expert Opin Investig Drugs*. 2006;15:1309-1318.
144. Mitchell MS, Abrams J, Thompson JA, et al. Randomized trial of an allogeneic melanoma lysate vaccine with low-dose interferon Alfa-2b compared with high-dose interferon Alfa-2b for resected stage III cutaneous melanoma. *J Clin Oncol*. 2007;25:2078-2085.
166. Slingluff Jr CL, Petroni GR, Yamshchikov GV, et al. Immunologic and clinical outcomes of vaccination with a multiepitope melanoma peptide vaccine plus low-dose interleukin-2 administered either concurrently or on a delayed schedule. *J Clin Oncol*. 2004;22:4474-4485.
169. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8$^+$ T cells in patients with melanoma. *J Immunol*. 2005;175:6169-6176.
170. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909-915.
171. Slingluff Jr CL, Petroni GR, Olson W, et al. Helper T-cell responses and clinical activity of a melanoma vaccine with multiple peptides from MAGE and melanocytic differentiation antigens. *J Clin Oncol*. 2008;26:4973-4980.
172. Welters MJ, Kenter GG, Piersma SJ, et al. Induction of tumor-specific CD4$^+$ and CD8$^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin Cancer Res*. 2008;14:178-187.
190. Mossoba ME, Medin JA. Cancer immunotherapy using virally transduced dendritic cells: animal studies and human clinical trials. *Expert Rev Vaccines*. 2006;5:717-732.
191. Su Z, Dannull J, Yang BK, et al. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8$^+$ and CD4$^+$ T cell responses in patients with metastatic prostate cancer. *J Immunol*. 2005;174:3798-3807.
192. Schadendorf D, Ugurel S, Schuler-Thurner B, et al. Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. *Ann Oncol*. 2006;17:563-570.
193. So-Rosillo R, Small EJ. Sipuleucel-T (APC8015) for prostate cancer. *Expert Rev Anticancer Ther*. 2006;6:1163-1167.
194. DeVita Jr VT. The Provenge decision. *Nat Clin Pract Oncol*. 2007;4:381.
209. Einstein MH, Kadish AS, Burk RD, et al. Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol Oncol*. 2007;106:453-460.
210. Aalamian M, Fuchs E, Gupta R, et al. Autologous renal cell cancer vaccines using heat shock protein-peptide complexes. *Urol Oncol*. 2006;24:425-433.
221. Garnett CT, Greiner JW, Tsang KY, et al. TRICOM vector based cancer vaccines. *Curr Pharm Des*. 2006;12:351-361.
222. Brockstedt DG, Giedlin MA, Leong ML, et al. Listeria-based cancer vaccines that segregate immunogenicity from toxicity. *Proc Natl Acad Sci U S A*. 2004;101:13832-13837.
225. Brockstedt DG, Bahjat KS, Giedlin MA, et al. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. *Nat Med*. 2005;11:853-860.
226. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363:411-422.
227. Kantoff PW, Schuetz TJ, Blumenstein BA, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol*. 2010;28:1099-1105.
228. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364:2119-2127.
229. Le DT, Wang-Gillam A, Picozzi VJ, et al. Interim safety and efficacy analysis of a phase II, randomized study of GVAX pancreas and CRS-207 immunotherapy in patients with metastatic pancreatic cancer. *J Clin Oncol*. 2013;31 (suppl; abstr 4040^).
230. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*. 2009;361:1838-1847.
232. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev*. 2009;229:12-26.
241. Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol*. 2005;25:9543-9553.
243. Schneider H, Downey J, Smith A, et al. Reversal of the TCR stop signal by CTLA-4. *Science*. 2006;313:1972-1975.
245. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332:600-603.
248. Peggs KS, Quezada SA, Chambers CA, et al. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med*. 2009;206:1717-1725.
249. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3$^+$ regulatory T cell function. *Science*. 2008;322:271-275.
250. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat Immunol*. 2003;4:330-336.
251. Gavin MA, Rasmussen JP, Fontenot JD, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007;445:771-775.
252. Hill JA, Feuerer M, Tash K, et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity*. 2007;27:786-800.
255. Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 2003;100:4712-4717.
256. Beck KE, Blansfield JA, Tran KQ, et al. Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte-associated antigen 4. *J Clin Oncol*. 2006;24:2283-2289.
257. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003;100:8372-8377.
258. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol*. 2005;23:8968-8977.
259. Ribas A. Clinical development of the anti-CTLA-4 antibody tremelimumab. *Semin Oncol*. 2010;37:450-454.
260. Downey SG, Klapper JA, Smith FO, et al. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res*. 2007;13:6681-6688.
261. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.

262. Ji RR, Chasalow SD, Wang L, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother*. 2012;61:1019-1031.
263. Liakou CI, Kamat A, Tang DN, et al. CTLA-4 blockade increases IFNgamma-producing CD4+ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A*. 2008;105:14987-14992.
264. Yuan J, Adamow M, Ginsberg BA, et al. Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A*. 2011;108:16723-16728.
265. Hoos A, Eggermont AM, Janetzki S, et al. Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst*. 2010;102:1388-1397.
268. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
269. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*. 2006;203:883-895.
272. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol*. 2007;19:813-824.
273. Blank C, Brown I, Peterson AC, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res*. 2004;64:1140-1145.
275. Taube JMAR, Xu H, Sharma R, et al. B7–H1 expression co-localizes with inflammatory infiltrates in benign and malignant melanocytic lesions: Implications for immunotherapy. *Sci Transl Med*. 2012 in press.
276. Fife BT, Pauken KE, Eagar TN, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol*. 2009;10:1185-1192.
277. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.
281. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*. 2007;27:111-122.
282. Park JJ, Omiya R, Matsumura Y, et al. B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood*. 2010;116:1291-1298.
283. Paterson AM, Brown KE, Keir ME, et al. The programmed death-1 ligand 1:B7-1 pathway restrains diabetogenic effector T cells in vivo. *J Immunol*. 2011;187:1097-1105.
284. Fanoni D, Tavecchio S, Recalcati S, et al. New monoclonal antibodies against B-cell antigens: possible new strategies for diagnosis of primary cutaneous B-cell lymphomas. *Immunol Lett*. 2011;134:157-160.
285. Terme M, Ullrich E, Aymeric L, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res*. 2011;71:5393-5399.
286. Velu V, Titanji K, Zhu B, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*. 2009;458:206-210.
287. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.
288. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*. 2009;114:1537-1544.
289. Sfanos KS, Bruno TC, Meeker AK, et al. Human prostate-infiltrating CD8+ T lymphocytes are oligoclonal and PD-1+. *Prostate*. 2009;69:1694-1703.
290. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol*. 2008;8:467-477.
291. Konishi J, Yamazaki K, Azuma M, et al. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res*. 2004;10:5094-5100.
292. Brown JA, Dorfman DM, Ma FR, et al. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol*. 2003;170:1257-1266.
293. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med*. 2003;9:562-567.
294. Kuang DM, Zhao Q, Peng C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med*. 2009;206:1327-1337.
295. Liu Y, Zeng B, Zhang Z, et al. B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin Immunol*. 2008;129:471-481.
296. Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A*. 2004;101:17174-17179.
297. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia*. 2006;8:190-198.
298. Hamanishi J, Mandai M, Iwasaki M, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A*. 2007;104:3360-3365.
299. Hino R, Kabashima K, Kato Y, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer*. 2010;116:1757-1766.
300. Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res*. 2005;11:2947-2953.
301. Wu C, Zhu Y, Jiang J, et al. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem*. 2006;108:19-24.
302. Steidl C, Shah SP, Woolcock BW, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471:377-381.
303. Parsa AT, Waldron JS, Panner A, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med*. 2007;13:84-88.
304. Marzec M, Zhang Q, Goradia A, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A*. 2008;105:20852-20857.
305. Kim J, Myers AC, Chen L, et al. Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2005;33:280-289.
306. Lee SK, Seo SH, Kim BS, et al. IFN-gamma regulates the expression of B7-H1 in dermal fibroblast cells. *J Dermatol Sci*. 2005;40:95-103.
307. Wilke CM, Wei S, Wang L, et al. Dual biological effects of the cytokines interleukin-10 and interferon-gamma. *Cancer Immunol Immunother*. 2011;60:1529-1541.
308. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer J*. 2010;16:399-403.
309. Brahmer JR, Drake CG, Wollner I, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010;28:3167-3175.
310. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
311. Topalian SL, Sznol M, Brahmer JR, et al. Nivolumab (anti-PD-1; BMS-936558; ONO-4538) in patients with advanced solid tumors: survival and long-term safety in a phase I trial. *J Clin Oncol*. 2013;31 (suppl; abstr 3002^).
312. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
313. Hamid O, Robert C, Daud A, et al. Safety and tumor responses with lambrolizumab (anti-pd-1) in melanoma. *N Engl J Med*. 2013;369:134-144.
314. Herbst RS, Gordon MS, Fine GD, et al. A study of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors. *J Clin Oncol*. 2013;31 (suppl; abstr 3000).
315. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*. 2013;369:122-133.
316. Yi KH, Chen L. Fine tuning the immune response through B7-H3 and B7-H4. *Immunol Rev*. 2009;229:145-151.
317. He C, Qiao H, Jiang H, et al. The inhibitory role of b7-h4 in antitumor immunity: association with cancer progression and survival. *Clin Dev Immunol*. 2011:695834, 2011.
319. Goldberg MV, Drake CG. LAG-3 in cancer immunotherapy. *Curr Top Microbiol Immunol*. 2011;344:269-278.

320. Grosso JF, Kelleher CC, Harris TJ, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest*. 2007;117:3383-3392.
321. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol*. 2009;10:29-37.
322. Grosso JF, Goldberg MV, Getnet D, et al. Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. *J Immunol*. 2009;182:6659-6669.
323. Woo SR, Turnis ME, Goldberg MV, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res*. 2012;72:917-927.
324. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. 2005;6:1245-1252.
325. Ngiow SF, von Scheidt B, Akiba H, et al. Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. *Cancer Res*. 2011;71:3540-3551.
326. Baitsch L, Legat A, Barba L, et al. Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. *PLoS One*. 2012;7:e30852.
327. Fourcade J, Sun Z, Benallaoua M, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. 2010;207:2175-2186.
328. Sakuishi K, Apetoh L, Sullivan JM, et al. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med*. 2010;207:2187-2194.
329. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol*. 2003;4:670-679.
330. Sedy JR, Gavrieli M, Potter KG, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol*. 2005;6:90-98.
331. Lasaro MO, Sazanovich M, Giles-Davis W, et al. Active immunotherapy combined with blockade of a coinhibitory pathway achieves regression of large tumor masses in cancer-prone mice. *Mol Ther*. 2011;19:1727-1736.
332. Fourcade J, Sun Z, Pagliano O, et al. CD8+ T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res*. 2012;72:889-896.
333. Zarek PE, Huang CT, Lutz ER, et al. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood*. 2008;111:251-259.
334. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007;204:1257-1265.
335. Waickman AT, Alme A, Senaldi L, et al. Enhancement of tumor immunotherapy by deletion of the A2A adenosine receptor. *Cancer Immunol Immunother*. 2012;61:917-926.
336. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. 2008;9:495-502.
337. Plougastel BF, Yokoyama WM. Extending missing-self? Functional interactions between lectin-like NKrp1 receptors on NK cells with lectin-like ligands. *Curr Top Microbiol Immunol*. 2006;298:77-89.
338. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. *Semin Immunol*. 2006;18:145-150.
339. Mingari MC, Pietra G, Moretta L. Human cytolytic T lymphocytes expressing HLA class-I-specific inhibitory receptors. *Curr Opin Immunol*. 2005;17:312-319.

This page intentionally left blank

References

1. Baldwin RW. Immunity to methylcholanthrene-induced tumours in inbred rats following atrophy and regression of the implanted tumours. *Br J Cancer*. 1955;9:652-657.
2. Foley EJ. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res*. 1953;13:835-837.
3. Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res*. 1943;3:326-333.
4. Old LJ, Carswell EA, Boyse EA, et al. Antigenic properties of chemically induced tumors. *Ann N Y Acad Sci*. 1962;101:80-106.
5. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst*. 1957;18:769-778.
6. Ciardiello F, Bianco R, Damiano V, et al. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res*. 1999;5:909-916.
7. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
8. Bronner CE, Baker SM, Morrison PT, et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature*. 1994;368:258-261.
9. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990;61:759-767.
10. Fishel R, Lescoe MK, Rao MRS, et al. The human mutator gene homolog Msh2 and its association with hereditary nonpolyposis colon-cancer. *Cell*. 1993;75:1027-1038.
11. Gowen LC, Avrutskaya AV, Latour AM, et al. BRCA1 required for transcription-coupled repair of oxidative DNA damage. [Retracted article. See vol. 300, p 1657, June 13, 2003] . *Science*. 1998;281:1009-1012.
12. Leach FS, Nicolaides NC, Papadopoulos N, et al. Mutations of a Muts homolog in hereditary nonpolyposis colorectal-cancer. *Cell*. 1993;75:1215-1225.
13. Lu X, Lane DP. Differential induction of transcriptionally active P53 Following UV or ionizing-radiation—defects in chromosome instability syndromes. *Cell*. 1993;75:765-778.
14. Papadopoulos N, Nicolaides NC, Wei YF, et al. Mutation of a mutL homolog in hereditary colon cancer. *Science*. 1994;263:1625-1629.
15. Sharan SK, Morimatsu M, Albrecht U, et al. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature*. 1997;386:804-810.
16. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science*. 2006;314:268-274.
17. Almoguera C, Shibata D, Forrester K, et al. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell*. 1988;53:549-554.
18. Bressac B, Kew M, Wands J, et al. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature*. 1991;350:429-431.
19. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.
20. Forrester K, Almoguera C, Han K, et al. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature*. 1987;327:298-303.
21. Abrams SI, Khleif SN, Bergmann-Leitner ES, et al. Generation of stable CD4$^+$ and CD8$^+$ T cell lines from patients immunized with ras oncogene-derived peptides reflecting codon 12 mutations. *Cell Immunol*. 1997;182:137-151.
22. Gjertsen MK, Bjorheim J, Saeterdal I, et al. Cytotoxic CD4$^+$ and CD8$^+$ T lymphocytes, generated by mutant p21-ras (12Val) peptide vaccination of a patient, recognize 12Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. *Int J Cancer*. 1997;72:784-790.
23. Sharkey MS, Lizee G, Gonzales MI, et al. CD4$^+$ T-cell recognition of mutated B-RAF in melanoma patients harboring the V599E mutation. *Cancer Res*. 2004;64:1595-1599.
24. Somasundaram R, Swoboda R, Caputo L, et al. Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. *Cancer Res*. 2006;66:3287-3293.
25. Jones PA, Baylin SB. The epigenomics of cancer. *Cell*. 2007;128:683-692.
26. Thomas AM, Santarsiero LM, Lutz ER, et al. Mesothelin-specific CD8$^+$ T cell responses provide evidence of in vivo cross-priming by antigen-presenting cells in vaccinated pancreatic cancer patients. *J Exp Med*. 2004;200:297-306.
27. Argani P, Iacobuzio-Donahue C, Ryu B, et al. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res*. 2001;7:3862-3868.
28. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A*. 1996;93:136-140.
29. Van Der Bruggen P, Zhang Y, Chaux P, et al. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev*. 2002;188:51-64.
30. Madsen B, Tarsounas M, Burchell JM, et al. PLU-1, a transcriptional repressor and putative testis-cancer antigen, has a specific expression and localisation pattern during meiosis. *Chromosoma*. 2003;112:124-132.
31. Osterlund C, Tohonen V, Forslund KO, et al. Mage-b4, a novel melanoma antigen (MAGE) gene specifically expressed during germ cell differentiation. *Cancer Res*. 2000;60:1054-1061.
32. Tureci O, Sahin U, Zwick C, et al. Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. *Proc Natl Acad Sci U S A*. 1998;95:5211-5216.
33. Brichard V, Vanpel A, Wolfel T, et al. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T-lymphocytes on Hla-A2 melanomas. *J Exp Med*. 1993;178:489-495.
34. Topalian SL, Rivoltini L, Mancini M, et al. Human CD4$^+$ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. *Proc Natl Acad Sci U S A*. 1994;91:9461-9465.
35. Anichini A, Maccalli C, Mortarini R, et al. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. *J Exp Med*. 1993;177:989-998.
36. Kawakami Y, Robbins PF, Wang RF, et al. The use of melanosomal proteins in the immunotherapy of melanoma. *J Immunother*. 1998;21:237-246.
37. Lutz E, Yeo CJ, Lillemoe KD, et al. A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation. *Ann Surg*. 2011;253:328-335.
38. Burnet FM. The concept of immunological surveillance. *Prog Exp Tumor Res*. 1970;13:1-27.
39. Thomas L. Discussion. In: Lawrence HS, ed. *Cellular and Humoral Aspects of the Hypersensitive States*. New York, NY: Hoeber-Harper; 1959.
40. Holland JM, Mitchell TJ, Gipson LC, et al. Survival and cause of death in aging germfree athymic nude and normal inbred C3Hf/He mice. *J Natl Cancer Inst*. 1978;61:1357-1361.
41. Moller G, Moller E. The concept of immunological surveillance against neoplasia. *Transplant Rev*. 1976;28:3-16.
42. Outzen HC, Custer RP, Eaton GJ, et al. Spontaneous and induced tumor incidence in germfree "nude" mice. *J Reticuloendothel Soc*. 1975;17:1-9.
43. Rygaard J, Povlsen CO. The nude mouse vs. the hypothesis of immunological surveillance. *Transplant Rev*. 1976;28:43-61.
44. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science*. 1974;183:534-536.
45. Gaidano G, Dalla-Favera R. Biologic aspects of human immunodeficiency virus-related lymphoma. *Curr Opin Oncol*. 1992;4:900-906.
46. Frizzera G. Atypical lymphoproliferative disorders. In: Knowles DM, ed. *Neoplastic Hematopathology*. Baltimore, Md: Williams & Wilkins; 1992:459-495.
47. Penn I. Tumors of the immunocompromised patient. *Annu Rev Med*. 1988;39:63-73.
48. Heslop HE, Ng CY, Li C, et al. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med*. 1996;2:551-555.
49. Mesri EA, Cesarman E, Arvanitakis L, et al. Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. *J Exp Med*. 1996;183:2385-2390.

50. Beaudenon S, Kremsdorf D, Obalek S, et al. Plurality of genital human papillomaviruses: characterization of two new types with distinct biological properties. *Virology*. 1987;161:374-384.
51. Boshart M, Gissmann L, Ikenberg H, et al. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J*. 1984;3:1151-1157.
52. McFarlane GA, Munro A. *Helicobacter pylori* and gastric cancer. *Br J Surg*. 1997;84:1190-1199.
53. Euvrard S, Kanitakis J, Pouteil-Noble C, et al. Skin cancers in organ transplant recipients. *Ann Transplant*. 1997;2:28-32.
54. Fairman RM, Grossman RA, Barker CF, et al. Inadvertent transplantation of a melanoma. *Transplantation*. 1980;30:328-330.
55. Jeremy D, Farnsworth RH, Robertson MR, et al. Transplantation of malignant melanoma with cadaver kidney. *Transplantation*. 1972;13:619-620.
56. Peters MS, Stuard ID. Metastatic malignant melanoma transplanted via a renal homograft: a case report. *Cancer*. 1978;41:2426-2430.
57. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6:836-848.
58. Fenner JE, Starr R, Cornish AL, et al. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat Immunol*. 2006;7:33-39.
59. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410:1107-1111.
60. Enzler TD, G: Increased tumor incidence in GM-CSF/IL-3/gamma-IFN knockout mice, 2002.
61. DuPage M, Mazumdar C, Schmidt LM, et al. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature*. 2012;482:405-409.
62. Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature*. 2005;437:141-146.
63. Koebel CM, Vermi W, Swann JB, et al. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*. 2007;450:903-907.
64. Bogen B. Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4$^+$ T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. *Eur J Immunol*. 1996;26:2671-2679.
65. Bogen B, Munthe L, Sollien A, et al. Naive CD4$^+$ T cells confer idiotype-specific tumor resistance in the absence of antibodies. *Eur J Immunol*. 1995;25:3079-3086.
66. Sotomayor EM, Borrello I, Rattis FM, et al. Cross-presentation of tumor antigens by bone marrow-derived antigen-presenting cells is the dominant mechanism in the induction of T-cell tolerance during B-cell lymphoma progression. *Blood*. 2001;98:1070-1077.
67. Staveley-O'Carroll K, Sotomayor E, Montgomery J, et al. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci U S A*. 1998;95:1178-1183.
68. Speiser DE, Miranda R, Zakarian A, et al. Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J Exp Med*. 1997;186:645-653.
69. Wick M, Dubey P, Koeppen H, et al. Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. *J Exp Med*. 1997;186:229-238.
70. Drake CG, Doody AD, Mihalyo MA, et al. Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. *Cancer Cell*. 2005;7:239-249.
71. Nguyen LT, Elford AR, Murakami K, et al. Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. *J Exp Med*. 2002;195:423-435.
72. Deng L, Langley RJ, Brown PH, et al. Structural basis for the recognition of mutant self by a tumor-specific, MHC class II-restricted T cell receptor. *Nat Immunol*. 2007;8:398-408.
73. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3$^+$ CD25$^+$ CD4$^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27.
74. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155:1151-1164.
75. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med*. 2004;10:942-949.
76. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*. 2002;169:2756-2761.
77. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299:1057-1061.
78. Bruder D, Probst-Kepper M, Westendorf AM, et al. Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol*. 2004;34:623-630.
79. Huang CT, Workman CJ, Flies D, et al. Role of LAG-3 in regulatory T cells. *Immunity*. 2004;21:503-513.
80. McHugh RS, Whitters MJ, Piccirillo CA, et al. CD4$^+$D25$^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*. 2002;16:311-323.
81. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*. 2007;8:277-284.
82. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol*. 2007;8:457-462.
83. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25$^+$ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med*. 2001;194:823-832.
84. Berd D, Maguire Jr HC, Mastrangelo MJ. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. *Cancer Res*. 1986;46:2572-2577.
85. Berd D, Mastrangelo MJ, Engstrom PF, et al. Augmentation of the human immune response by cyclophosphamide. *Cancer Res*. 1982;42:4862-4866.
86. Ercolini AM, Ladle BH, Manning EA, et al. Recruitment of latent pools of high-avidity CD8$^+$ T cells to the antitumor immune response. *J Exp Med*. 2005;201:1591-1602.
87. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med*. 1982;155:1063-1074.
88. Telang S, Rasku MA, Clem AL, et al. Phase II trial of the regulatory T cell-depleting agent, denileukin diftitox, in patients with unresectable stage IV melanoma. *BMC Cancer*. 2011;11:515.
89. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother*. 2006;55:237-245.
90. Young MR, Petruzzelli GJ, Kolesiak K, et al. Human squamous cell carcinomas of the head and neck chemoattract immune suppressive CD34$^+$ progenitor cells. *Hum Immunol*. 2001;62:332-341.
91. Bronte V, Apolloni E, Cabrelle A, et al. Identification of a CD11b$^+$Gr-1$^+$CD31$^+$ myeloid progenitor capable of activating or suppressing CD8$^+$ T cells. *Blood*. 2000;96:3838-3846.
92. Bronte V, Serafini P, De Santo C, et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J Immunol*. 2003;170:270-278.
93. Mazzoni A, Bronte V, Visintin A, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol*. 2002;168:689-695.
94. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res*. 2005;65:3044-3048.
95. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res*. 2001;61:4756-4760.
96. Munn DH, Sharma MD, Lee JR, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science*. 2002;297:1867-1870.
97. Baban B, Hansen AM, Chandler PR, et al. A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type I IFN signaling following B7 ligation. *Int Immunol*. 2005;17:909-919.
98. Mellor AL, Chandler P, Baban B, et al. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3-dioxygenase. *Int Immunol*. 2004;16:1391-1401.

99. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest*. 2004;114:280-290.
100. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med*. 2003;9:1269-1274.
101. Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*. 2011;478:197-203.
102. Mezrich JD, Fechner JH, Zhang X, et al. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol*. 2010;185:3190-3198.
103. Favre D, Mold J, Hunt PW, et al. Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med*. 2010;2:32ra36.
104. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med*. 2000;342:1350-1358.
105. Pepper MS. Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev*. 1997;8:21-43.
106. Cui W, Fowlis DJ, Bryson S, et al. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell*. 1996;86:531-542.
107. Shariat SF, Shalev M, Menesses-Diaz A, et al. Preoperative plasma levels of transforming growth factor beta^1 (TGF-beta(1)) strongly predict progression in patients undergoing radical prostatectomy. *J Clin Oncol*. 2001;19:2856-2864.
108. Hasegawa Y, Takanashi S, Kanehira Y, et al. Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer*. 2001;91:964-971.
109. Saito H, Tsujitani S, Oka S, et al. The expression of transforming growth factor-beta1 is significantly correlated with the expression of vascular endothelial growth factor and poor prognosis of patients with advanced gastric carcinoma. *Cancer*. 1999;86:1455-1462.
110. Shariat SF, Kim JH, Andrews B, et al. Preoperative plasma levels of transforming growth factor beta(1) strongly predict clinical outcome in patients with bladder carcinoma. *Cancer*. 2001;92:2985-2992.
111. Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annu Rev Immunol*. 1998;16:137-161.
112. Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity*. 2000;12:171-181.
113. Chen ML, Pittet MJ, Gorelik L, et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A*. 2005;102:419-424.
114. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245-252.
115. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.
116. Caux C, Dezutter-Dambuyant C, Schmitt D, et al. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature*. 1992;360:258-261.
117. Inaba K, Steinman RM, Pack MW, et al. Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med*. 1992;175:1157-1167.
118. Kiertscher SM, Roth MD. Human CD14$^+$ leukocytes acquire the phenotype and function of antigen-presenting dendritic cells when cultured in GM-CSF and IL-4. *J Leukoc Biol*. 1996;59:208-218.
119. Maraskovsky E, Brasel K, Teepe M, et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med*. 1996;184:1953-1962.
120. Romani N, Reider D, Heuer M, et al. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J Immunol Methods*. 1996;196:137-151.
121. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. 2001;2:675-680.
122. Caux C, Massacrier C, Vanbervliet B, et al. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med*. 1994;180:1263-1272.
123. Cella M, Scheidegger D, Palmer-Lehmann K, et al. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med*. 1996;184:747-752.
124. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*. 1994;179:1109-1118.
125. Fritz JH, Ferrero RL, Philpott DJ, et al. Nod-like proteins in immunity, inflammation and disease. *Nat Immunol*. 2006;7:1250-1257.
126. Honda K, Sakaguchi S, Nakajima C, et al. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc Natl Acad Sci U S A*. 2003;100:10872-10877.
127. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*. 2006;441:101-105.
128. Cella M, Engering A, Pinet V, et al. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature*. 1997;388:782-787.
129. Pierre P, Turley SJ, Gatti E, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature*. 1997;388:787-792.
130. Berd D, Maguire Jr HC, McCue P, et al. Treatment of metastatic melanoma with an autologous tumor-cell vaccine: clinical and immunologic results in 64 patients. *J Clin Oncol*. 1990;8:1858-1867.
131. Hoover Jr HC, Brandhorst JS, Peters LC, et al. Adjuvant active specific immunotherapy for human colorectal cancer: 6.5-year median follow-up of a phase III prospectively randomized trial. *J Clin Oncol*. 1993;11:390-399.
132. Hoover Jr HC, Surdyke MG, Dangel RB, et al. Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. *Cancer*. 1985;55:1236-1243.
133. Lipton A, Harvey HA, Balch CM, et al. *Corynebacterium parvum* versus bacille Calmette-Guerin adjuvant immunotherapy of stage II malignant melanoma. *J Clin Oncol*. 1991;9:1151-1156.
134. McCune CS, O'Donnell RW, Marquis DM, et al. Renal cell carcinoma treated by vaccines for active specific immunotherapy: correlation of survival with skin testing by autologous tumor cells. *Cancer Immunol Immunother*. 1990;32:62-66.
135. Berd D. Autologous, hapten-modified vaccine as a treatment for human cancers. *Vaccine*. 2001;19:2565-2570.
136. Schirrmacher V, Haas C, Bonifer R, et al. Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle disease virus. *Gene Ther*. 1999;6:63-73.
137. Morton DL, Foshag LJ, Nizze JA, et al. Active specific immunotherapy in malignant melanoma. *Semin Surg Oncol*. 1989;5:420-425.
138. Hanna Jr MG, Hoover Jr HC, Vermorken JB, et al. Adjuvant active specific immunotherapy of stage II and stage III colon cancer with an autologous tumor cell vaccine: first randomized phase III trials show promise. *Vaccine*. 2001;19:2576-2582.
139. Morton DL, Hsueh EC, Essner R, et al. Prolonged survival of patients receiving active immunotherapy with Canvaxin therapeutic polyvalent vaccine after complete resection of melanoma metastatic to regional lymph nodes. *Ann Surg*. 2002;236:438-448; discussion 448-449.
140. Harris JE, Ryan L, Hoover Jr HC, et al. Adjuvant active specific immunotherapy for stage II and III colon cancer with an autologous tumor cell vaccine: Eastern Cooperative Oncology Group Study E5283. *J Clin Oncol*. 2000;18:148-157.
141. Kelland L. Discontinued drugs in 2005: oncology drugs. *Expert Opin Investig Drugs*. 2006;15:1309-1318.
142. Sondak VK, Liu PY, Tuthill RJ, et al. Adjuvant immunotherapy of resected, intermediate-thickness, node-negative melanoma with an allogeneic tumor vaccine: overall results of a randomized trial of the Southwest Oncology Group. *J Clin Oncol*. 2002;20:2058-2066.
143. Berd D, Sato T, Cohn H, et al. Treatment of metastatic melanoma with autologous, hapten-modified melanoma vaccine: regression of pulmonary metastases. *Int J Cancer*. 2001;94:531-539.
144. Mitchell MS, Abrams J, Thompson JA, et al. Randomized trial of an allogeneic melanoma lysate vaccine with low-dose interferon Alfa-2b compared with high-dose interferon Alfa-2b for resected stage III cutaneous melanoma. *J Clin Oncol*. 2007;25:2078-2085.

145. Lindenmann J, Klein PA. Viral oncolysis: increased immunogenicity of host cell antigen associated with influenza virus. *J Exp Med*. 1967;126:93-108.
146. Fearon ER, Itaya T, Hunt B, et al. Induction in a murine tumor of immunogenic tumor variants by transfection with a foreign gene. *Cancer Res*. 1988;48:2975-2980.
147. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol*. 1995;13:399-415.
148. Borrello I, Sotomayor EM, Cooke S, et al. A universal granulocyte-macrophage colony-stimulating factor-producing bystander cell line for use in the formulation of autologous tumor cell-based vaccines. *Hum Gene Ther*. 1999;10:1983-1991.
149. Fearon ER, Pardoll DM, Itaya T, et al. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell*. 1990;60:397-403.
150. Gansbacher B, Zier K, Daniels B, et al. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med*. 1990;172:1217-1224.
151. Dranoff G, Jaffee E, Lazenby A, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A*. 1993;90:3539-3543.
152. Golumbek PT, Lazenby AJ, Levitsky HI, et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science*. 1991;254:713-716.
153. Porgador A, Tzehoval E, Katz A, et al. Interleukin 6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metastatic cells. *Cancer Res*. 1992;52:3679-3686.
154. Hung K, Hayashi R, Lafond-Walker A, et al. The central role of CD4+ T cells in the antitumor immune response. *J Exp Med*. 1998;188:2357-2368.
155. Simons JW, Jaffee EM, Weber CE, et al. Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. *Cancer Res*. 1997;57:1537-1546.
156. Feltkamp MC, Smits HL, Vierboom MP, et al. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol*. 1993;23:2242-3349.
157. Kast WM, Roux L, Curren J, et al. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. *Proc Natl Acad Sci U S A*. 1991;88:2283-2287.
158. Schulz M, Zinkernagel RM, Hengartner H. Peptide-induced antiviral protection by cytotoxic T cells. *Proc Natl Acad Sci U S A*. 1991;88:991-993.
159. Mandelboim O, Vadai E, Fridkin M, et al. Regression of established murine carcinoma metastases following vaccination with tumour-associated antigen peptides. *Nat Med*. 1995;1:1179-1183.
160. Aichele P, Brduscha-Riem K, Zinkernagel RM, et al. T cell priming versus T cell tolerance induced by synthetic peptides. *J Exp Med*. 1995;182:261-266.
161. Aichele P, Kyburz D, Ohashi PS, et al. Peptide-induced T-cell tolerance to prevent autoimmune diabetes in a transgenic mouse model. *Proc Natl Acad Sci U S A*. 1994;91:444-448.
162. Toes RE, Blom RJ, Offringa R, et al. Enhanced tumor outgrowth after peptide vaccination. Functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. *J Immunol*. 1996;156:3911-3918.
163. Zwaveling S, Ferreira Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol*. 2002;169:350-358.
164. Schild H, Norda M, Deres K, et al. Fine specificity of cytotoxic T lymphocytes primed in vivo either with virus or synthetic lipopeptide vaccine or primed in vitro with peptide. *J Exp Med*. 1991;174:1665-1668.
165. Vitiello A, Ishioka G, Grey HM, et al. Development of a lipopeptide-based therapeutic vaccine to treat chronic HBV infection. I. Induction of a primary cytotoxic T lymphocyte response in humans. *J Clin Invest*. 1995;95:341-349.
166. Slingluff Jr CL, Petroni GR, Yamshchikov GV, et al. Immunologic and clinical outcomes of vaccination with a multiepitope melanoma peptide vaccine plus low-dose interleukin-2 administered either concurrently or on a delayed schedule. *J Clin Oncol*. 2004;22:4474-4485.
167. Marchand M, Weynants P, Rankin E, et al. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. *Int J Cancer*. 1995;63:883-885.
168. Parkhurst MR, Salgaller ML, Southwood S, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J Immunol*. 1996;157:2539-2548.
169. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J Immunol*. 2005;175:6169-6176.
170. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med*. 2004;10:909-915.
171. Slingluff Jr CL, Petroni GR, Olson W, et al. Helper T-cell responses and clinical activity of a melanoma vaccine with multiple peptides from MAGE and melanocytic differentiation antigens. *J Clin Oncol*. 2008;26:4973-4980.
172. Welters MJ, Kenter GG, Piersma SJ, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin Cancer Res*. 2008;14:178-187.
173. Hsu FJ, Benike C, Fagnoni F, et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med*. 1996;2:52-58.
174. Lambert LA, Gibson GR, Maloney M, et al. Equipotent generation of protective antitumor immunity by various methods of dendritic cell loading with whole cell tumor antigens. *J Immunother*. 2001;24:232-236.
175. Mayordomo JI, Zorina T, Storkus WJ, et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat Med*. 1995;1:1297-1302.
176. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med*. 1998;4:328-332.
177. Paglia P, Chiodoni C, Rodolfo M, et al. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J Exp Med*. 1996;183:317-322.
178. Porgador A, Gilboa E. Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J Exp Med*. 1995;182:255-260.
179. Shimizu K, Thomas EK, Giedlin M, et al. Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein. *Cancer Res*. 2001;61:2618-2624.
180. Dyall J, Latouche JB, Schnell S, et al. Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. *Blood*. 2001;97:114-121.
181. Song W, Kong HL, Carpenter H, et al. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J Exp Med*. 1997;186:1247-1256.
182. Specht JM, Wang G, Do MT, et al. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J Exp Med*. 1997;186:1213-1221.
183. Boczkowski D, Nair SK, Snyder D, et al. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J Exp Med*. 1996;184:465-472.
184. Bender A, Sapp M, Schuler G, et al. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J Immunol Methods*. 1996;196:121-135.
185. Reddy A, Sapp M, Feldman M, et al. A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood*. 1997;90:3640-3646.
186. Dhodapkar MV, Steinman RM, Krasovsky J, et al. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med*. 2001;193:233-238.
187. Klein C, Bueler H, Mulligan RC. Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. *J Exp Med*. 2000;191:1699-1708.
188. Boyle JS, Brady JL, Lew AM. Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature*. 1998;392:408-411.
189. Gong J, Chen D, Kashiwaba M, et al. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat Med*. 1997;3:558-561.

190. Mossoba ME, Medin JA. Cancer immunotherapy using virally transduced dendritic cells: animal studies and human clinical trials. *Expert Rev Vaccines*. 2006;5:717-732.
191. Su Z, Dannull J, Yang BK, et al. Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. *J Immunol*. 2005;174:3798-3807.
192. Schadendorf D, Ugurel S, Schuler-Thurner B, et al. Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized phase III trial of the DC study group of the DeCOG. *Ann Oncol*. 2006;17:563-570.
193. So-Rosillo R, Small EJ. Sipuleucel-T (APC8015) for prostate cancer. *Expert Rev Anticancer Ther*. 2006;6:1163-1167.
194. DeVita Jr VT. The Provenge decision. *Nat Clin Pract Oncol*. 2007;4:381.
195. Blachere NE, Li Z, Chandawarkar RY, et al. Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med*. 1997;186:1315-1322.
196. Lammert E, Arnold D, Nijenhuis M, et al. The endoplasmic reticulum-resident stress protein gp96 binds peptides translocated by TAP. *Eur J Immunol*. 1997;27:923-927.
197. Srivastava PK. Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune response to cancer and in antigen presentation. *Adv Cancer Res*. 1993;62:153-177.
198. Srivastava PK, Heike M. Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? *Semin Immunol*. 1991;3:57-64.
199. Tamura Y, Peng P, Liu K, et al. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science*. 1997;278:117-120.
200. Udono H, Levey DL, Srivastava PK. Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells in vivo. *Proc Natl Acad Sci U S A*. 1994;91:3077-3081.
201. Udono H, Srivastava PK. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med*. 1993;178:1391-1396.
202. Udono H, Srivastava PK. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol*. 1994;152:5398-5403.
203. Suzue K, Zhou X, Eisen HN, et al. Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. *Proc Natl Acad Sci U S A*. 1997;94:13146-13151.
204. Chen CH, Wang TL, Hung CF, et al. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res*. 2000;60:1035-1042.
205. Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science*. 1995;269:1585-1588.
206. Basu S, Binder RJ, Ramalingam T, et al. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity*. 2001;14:303-313.
207. Asea A, Kraeft SK, Kurt-Jones EA, et al. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med*. 2000;6:435-442.
208. Kuppner MC, Gastpar R, Gelwer S, et al. The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *Eur J Immunol*. 2001;31:1602-1609.
209. Einstein MH, Kadish AS, Burk RD, et al. Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol Oncol*. 2007;106:453-460.
210. Aalamian M, Fuchs E, Gupta R, et al. Autologous renal cell cancer vaccines using heat shock protein-peptide complexes. *Urol Oncol*. 2006;24:425-433.
211. Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc Natl Acad Sci U S A*. 1996;93:11341-11348.
212. Panicali D, Davis SW, Weinberg RL, et al. Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proc Natl Acad Sci U S A*. 1983;80:5364-5368.
213. Smith GL, Murphy BR, Moss B. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc Natl Acad Sci U S A*. 1983;80:7155-7159.
214. Carroll MW, Overwijk WW, Chamberlain RS, et al. Highly attenuated modified vaccinia virus Ankara (MVA) as an effective recombinant vector: a murine tumor model. *Vaccine*. 1997;15:387-394.
215. Elzey BD, Siemens DR, Ratliff TL, et al. Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors. *Int J Cancer*. 2001;94:842-849.
216. Paoletti E, Taylor J, Meignier B, et al. Highly attenuated poxvirus vectors: NYVAC, ALVAC and TROVAC. *Dev Biol Stand*. 1995;84:159-163.
217. Velders MP, McElhiney S, Cassetti MC, et al. Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. *Cancer Res*. 2001;61:7861-7867.
218. Gewurz BE, Gaudet R, Tortorella D, et al. Virus subversion of immunity: a structural perspective. *Curr Opin Immunol*. 2001;13:442-450.
219. Irvine KR, Chamberlain RS, Shulman EP, et al. Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors. *J Natl Cancer Inst*. 1997;89:1595-1601.
220. Ramshaw IA, Ramsay AJ. The prime-boost strategy: exciting prospects for improved vaccination. *Immunol Today*. 2000;21:163-165.
221. Garnett CT, Greiner JW, Tsang KY, et al. TRICOM vector based cancer vaccines. *Curr Pharm Des*. 2006;12:351-361.
222. Brockstedt DG, Giedlin MA, Leong ML, et al. Listeria-based cancer vaccines that segregate immunogenicity from toxicity. *Proc Natl Acad Sci U S A*. 2004;101:13832-13837.
223. Darji A, Guzman CA, Gerstel B, et al. Oral somatic transgene vaccination using attenuated S. typhimurium. *Cell*. 1997;91:765-775.
224. Sizemore DR, Branstrom AA, Sadoff JC. Attenuated *Shigella* as a DNA delivery vehicle for DNA-mediated immunization. *Science*. 1995;270:299-302.
225. Brockstedt DG, Bahjat KS, Giedlin MA, et al. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. *Nat Med*. 2005;11:853-860.
226. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363:411-422.
227. Kantoff PW, Schuetz TJ, Blumenstein BA, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol*. 2010;28:1099-1105.
228. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364:2119-2127.
229. Le DT, Wang-Gillam A, Picozzi VJ, et al. Interim safety and efficacy analysis of a phase II, randomized study of GVAX pancreas and CRS-207 immunotherapy in patients with metastatic pancreatic cancer. *J Clin Oncol*. 2013;31 (suppl; abstr 4040^).
230. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*. 2009;361:1838-1847.
231. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol*. 1996;14:233-258.
232. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunol Rev*. 2009;229:12-26.
233. Schwartz RH. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell*. 1992;71:1065-1068.
234. Azuma M, Ito D, Yagita H, et al. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature*. 1993;366:76-79.
235. Freeman GJ, Gribben JG, Boussiotis VA, et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science*. 1993;262:909-911.
236. Hathcock KS, Laszlo G, Dickler HB, et al. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science*. 1993;262:905-907.
237. Linsley PS, Brady W, Urnes M, et al. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med*. 1991;174:561-569.
238. Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc Natl Acad Sci U S A*. 1990;87:5031-5035.

239. Egen JG, Allison JP. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity*. 2002;16:23-35.
240. Linsley PS, Greene JL, Brady W, et al. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity*. 1994;1:793-801.
241. Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol*. 2005;25:9543-9553.
242. Riley JL, Mao M, Kobayashi S, et al. Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc Natl Acad Sci U S A*. 2002;99:11790-11795.
243. Schneider H, Downey J, Smith A, et al. Reversal of the TCR stop signal by CTLA-4. *Science*. 2006;313:1972-1975.
244. Schneider H, Mandelbrot DA, Greenwald RJ, et al. Cutting edge: CTLA-4 (CD152) differentially regulates mitogen-activated protein kinases (extracellular signal-regulated kinase and c-Jun N-terminal kinase) in $CD4^+$ T cells from receptor/ligand-deficient mice. *J Immunol*. 2002;169:3475-3479.
245. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332:600-603.
246. Tivol EA, Borriello F, Schweitzer AN, et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 1995;3:541-547.
247. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science*. 1995;270:985-988.
248. Peggs KS, Quezada SA, Chambers CA, et al. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med*. 2009;206:1717-1725.
249. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over $Foxp3^+$ regulatory T cell function. *Science*. 2008;322:271-275.
250. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat Immunol*. 2003;4:330-336.
251. Gavin MA, Rasmussen JP, Fontenot JD, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007;445:771-775.
252. Hill JA, Feuerer M, Tash K, et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity*. 2007;27:786-800.
253. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science*. 1996;271:1734-1736.
254. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med*. 1999;190:355-366.
255. Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A*. 2003;100:4712-4717.
256. Beck KE, Blansfield JA, Tran KQ, et al. Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte-associated antigen 4. *J Clin Oncol*. 2006;24:2283-2289.
257. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A*. 2003;100:8372-8377.
258. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol*. 2005;23:8968-8977.
259. Ribas A. Clinical development of the anti-CTLA-4 antibody tremelimumab. *Semin Oncol*. 2010;37:450-454.
260. Downey SG, Klapper JA, Smith FO, et al. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res*. 2007;13:6681-6688.
261. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723.
262. Ji RR, Chasalow SD, Wang L, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother*. 2012;61:1019-1031.
263. Liakou CI, Kamat A, Tang DN, et al. CTLA-4 blockade increases IFNgamma-producing $CD4^+ICOShi$ cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proc Natl Acad Sci U S A*. 2008;105:14987-14992.
264. Yuan J, Adamow M, Ginsberg BA, et al. Integrated NY-ESO-1 antibody and $CD8^+$ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A*. 2011;108:16723-16728.
265. Hoos A, Eggermont AM, Janetzki S, et al. Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst*. 2010;102:1388-1397.
266. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 2000;192:1027-1034.
267. Ishida Y, Agata Y, Shibahara K, et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*. 1992;11:3887-3895.
268. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
269. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*. 2006;203:883-895.
270. Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 1999;11:141-151.
271. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 2001;291:319-322.
272. Okazaki T, Honjo T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol*. 2007;19:813-824.
273. Blank C, Brown I, Peterson AC, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic $CD8^+$ T cells. *Cancer Res*. 2004;64:1140-1145.
274. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 2002;8:793-800.
275. Taube JMAR, Xu H, Sharma R, et al. B7–H1 expression co-localizes with inflammatory infiltrates in benign and malignant melanocytic lesions: Implications for immunotherapy. *Sci Transl Med*. 2012; in press.
276. Fife BT, Pauken KE, Eagar TN, et al. Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. *Nat Immunol*. 2009;10:1185-1192.
277. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206:3015-3029.
278. Dong H, Zhu G, Tamada K, et al. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med*. 1999;5:1365-1369.
279. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*. 2001;2:261-268.
280. Tseng SY, Otsuji M, Gorski K, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med*. 2001;193:839-846.
281. Butte MJ, Keir ME, Phamduy TB, et al. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*. 2007;27:111-122.
282. Park JJ, Omiya R, Matsumura Y, et al. B7-H1/CD80 interaction is required for the induction and maintenance of peripheral T-cell tolerance. *Blood*. 2010;116:1291-1298.
283. Paterson AM, Brown KE, Keir ME, et al. The programmed death-1 ligand 1:B7-1 pathway restrains diabetogenic effector T cells in vivo. *J Immunol*. 2011;187:1097-1105.
284. Fanoni D, Tavecchio S, Recalcati S, et al. New monoclonal antibodies against B-cell antigens: possible new strategies for diagnosis of primary cutaneous B-cell lymphomas. *Immunol Lett*. 2011;134:157-160.
285. Terme M, Ullrich E, Aymeric L, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res*. 2011;71:5393-5399.
286. Velu V, Titanji K, Zhu B, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*. 2009;458:206-210.

287. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006;439:682-687.
288. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*. 2009;114:1537-1544.
289. Sfanos KS, Bruno TC, Meeker AK, et al. Human prostate-infiltrating CD8+ T lymphocytes are oligoclonal and PD-1+. *Prostate*. 2009;69:1694-1703.
290. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol*. 2008;8:467-477.
291. Konishi J, Yamazaki K, Azuma M, et al. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res*. 2004;10:5094-5100.
292. Brown JA, Dorfman DM, Ma FR, et al. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol*. 2003;170:1257-1266.
293. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med*. 2003;9:562-567.
294. Kuang DM, Zhao Q, Peng C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med*. 2009;206:1327-1337.
295. Liu Y, Zeng B, Zhang Z, et al. B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin Immunol*. 2008;129:471-481.
296. Thompson RH, Gillett MD, Cheville JC, et al. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A*. 2004;101:17174-17179.
297. Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-H1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia*. 2006;8:190-198.
298. Hamanishi J, Mandai M, Iwasaki M, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A*. 2007;104:3360-3365.
299. Hino R, Kabashima K, Kato Y, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer*. 2010;116:1757-1766.
300. Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res*. 2005;11:2947-2953.
301. Wu C, Zhu Y, Jiang J, et al. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem*. 2006;108:19-24.
302. Steidl C, Shah SP, Woolcock BW, et al. MHC class II transactivator CIITA is a recurrent gene fusion partner in lymphoid cancers. *Nature*. 2011;471:377-381.
303. Parsa AT, Waldron JS, Panner A, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med*. 2007;13:84-88.
304. Marzec M, Zhang Q, Goradia A, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A*. 2008;105:20852-20857.
305. Kim J, Myers AC, Chen L, et al. Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2005;33:280-289.
306. Lee SK, Seo SH, Kim BS, et al. IFN-gamma regulates the expression of B7-H1 in dermal fibroblast cells. *J Dermatol Sci*. 2005;40:95-103.
307. Wilke CM, Wei S, Wang L, et al. Dual biological effects of the cytokines interleukin-10 and interferon-gamma. *Cancer Immunol Immunother*. 2011;60:1529-1541.
308. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer J*. 2010;16:399-403.
309. Brahmer JR, Drake CG, Wollner I, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*. 2010;28:3167-3175.
310. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465.
311. Topalian SL, Sznol M, Brahmer JR, et al. Nivolumab (anti-PD-1; BMS-936558; ONO-4538) in patients with advanced solid tumors: survival and long-term safety in a phase I trial. *J Clin Oncol*. 2013;31 (suppl; abstr 3002^).
312. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454.
313. Hamid O, Robert C, Daud A, et al. Safety and tumor responses with lambrolizumab (anti-pd-1) in melanoma. *N Engl J Med*. 2013;369:134-144.
314. Herbst RS, Gordon MS, Fine GD, et al. A study of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic tumors. *J Clin Oncol*. 2013;31 (suppl; abstr 3000).
315. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med*. 2013;369:122-133.
316. Yi KH, Chen L. Fine tuning the immune response through B7-H3 and B7-H4. *Immunol Rev*. 2009;229:145-151.
317. He C, Qiao H, Jiang H, et al. The inhibitory role of b7-h4 in antitumor immunity: association with cancer progression and survival. *Clin Dev Immunol*. 2011:695834, 2011.
318. Triebel F, Jitsukawa S, Baixeras E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med*. 1990;171:1393-1405.
319. Goldberg MV, Drake CG. LAG-3 in cancer immunotherapy. *Curr Top Microbiol Immunol*. 2011;344:269-278.
320. Grosso JF, Kelleher CC, Harris TJ, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest*. 2007;117:3383-3392.
321. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol*. 2009;10:29-37.
322. Grosso JF, Goldberg MV, Getnet D, et al. Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. *J Immunol*. 2009;182:6659-6669.
323. Woo SR, Turnis ME, Goldberg MV, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res*. 2012;72:917-927.
324. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. 2005;6:1245-1252.
325. Ngiow SF, von Scheidt B, Akiba H, et al. Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. *Cancer Res*. 2011;71:3540-3551.
326. Baitsch L, Legat A, Barba L, et al. Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. *PLoS One*. 2012;7:e30852.
327. Fourcade J, Sun Z, Benallaoua M, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. 2010;207:2175-2186.
328. Sakuishi K, Apetoh L, Sullivan JM, et al. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med*. 2010;207:2187-2194.
329. Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol*. 2003;4:670-679.
330. Sedy JR, Gavrieli M, Potter KG, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol*. 2005;6:90-98.
331. Lasaro MO, Sazanovich M, Giles-Davis W, et al. Active immunotherapy combined with blockade of a coinhibitory pathway achieves regression of large tumor masses in cancer-prone mice. *Mol Ther*. 2011;19:1727-1736.
332. Fourcade J, Sun Z, Pagliano O, et al. CD8+ T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res*. 2012;72:889-896.
333. Zarek PE, Huang CT, Lutz ER, et al. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood*. 2008;111:251-259.

334. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*. 2007;204:1257-1265.
335. Waickman AT, Alme A, Senaldi L, et al. Enhancement of tumor immunotherapy by deletion of the A2A adenosine receptor. *Cancer Immunol Immunother*. 2012;61:917-926.
336. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. 2008;9:495-502.
337. Plougastel BF, Yokoyama WM. Extending missing-self? Functional interactions between lectin-like NKrp1 receptors on NK cells with lectin-like ligands. *Curr Top Microbiol Immunol*. 2006;298:77-89.
338. Raulet DH. Missing self recognition and self tolerance of natural killer (NK) cells. *Semin Immunol*. 2006;18:145-150.
339. Mingari MC, Pietra G, Moretta L. Human cytolytic T lymphocytes expressing HLA class-I-specific inhibitory receptors. *Curr Opin Immunol*. 2005;17:312-319.

Ernest C. Borden*

53 Interferons

Introduction

Interferons (IFNs) comprise a family of secreted α-helical cytokines induced in response to specific extracellular biomolecules through stimulation of Toll-like receptors (TLRs). Acting in paracrine or autocrine modes, IFNs stimulate intra- and intercellular networks for regulating innate and acquired immunity, resistance to viral infections, and normal and tumor cell survival and death (Table 53-1). Through high-affinity cell surface receptors, IFNs stimulate genes, employing signaling molecules also used in part by other cytokines first identified through studies of IFNs. Perturbations in these pathways can also make cells resistant to a given ligand, facilitating either progression or resistance of malignancy. IFNs act on almost every cell type and, through their cellular actions, can be effective in inhibiting tumor emergence and progression and in inducing regression (see Table 53-1).

Studies on the mechanisms by which IFNs exert their antitumor activity have helped to understand host resistance to tumor emergence and also define cellular actions of interferon-stimulated gene (ISG) products (see Table 53-1). These latter proteins underlie not only the antitumor and immunoregulatory actions but also the antiviral effects of IFNs. More than a thousand genes regulated through IFN signaling pathways now have been identified.[1,2] Suppression of IFNs and influences on their regulated gene products in and by malignant cells is emerging as an important contributor to the development of some human cancers (Table 53-2). Germ-cell mutation of an ISG *RNASEL* increases risk for prostate, breast, head and neck, and pancreatic carcinomas.[3-6] Gene expression profiling and cytogenetic analyses have identified somatic homozygous deletions in the locus for IFNs at 9p21 and mutations of ISGs in melanoma, colon, lung, and hematologic malignancies.[7-12] Epigenetic and genetic silencing of IFN signaling also likely influences tumor development.[11,13-15] Activated natural killer (NK) and T cells have a critical role in the production and action of IFNs for potent immunomodulatory roles in the protection from chemical carcinogenesis and in controlling the growth of syngeneic and transplanted tumors. In addition to being a primary source for the production of IFNs-α and IFN-β, dendritic cell maturation is also influenced by IFNs.[16-19] These actions of endogenous IFNs-α, IFN-β, and IFN-γ are probably the basis for the effectiveness of IFNs and/or inducers in suppressing tumor emergence and progression.[20-22] These immunomodulatory actions, however, may or may not be identical to those resulting in clinical tumor regression with IFNs administered as single agents or in conjunction with other modalities of therapy.

Before the development of recombinant DNA technology for protein synthesis in prokaryotes, only limited quantities of impure IFNs were available. IFNs were, indeed, the first proteins produced by recombinant technology that were not previously available for wide use in clinical medicine. A long-awaited milestone, U.S. Food and Drug Administration (FDA) approval for a defined human protein with potent cell regulatory effects for the treatment of human malignancy, was subsequently realized.[23,24] IFNs thus became the prototypic biologic response modifiers for clinical oncologic therapy. With an emphasis on studies in human cells, this chapter reviews the structure of this family of cytokines, receptor interactions, signal transduction pathways, mechanisms of action, the ISGs, and clinical antitumor activity.

Induction, Genes, Receptors, and Signaling

Interferon Genes, Proteins, and Their Induction

There are several types and families of IFNs, all of which have antiviral effects. Classification of IFNs is based on primary

*Supported in part by NIH P30 CA043703.

Table 53-1 Regulatory Molecules in the Antitumor Mechanism of Action of Interferons

Induction
TLRs MyD88, RIG-I IRFs
Receptors
IFNAR1, IFNAR2 IFNGR IFNLR1, IL-10R2
Signal Transduction
JAKs, STATs IRFs PI3K
Interferon-Stimulated Genes (ISGs)
Cellular Effects
Apoptosis Immunoregulatory Anti-angiogenic

Table 53-2 IFNs in Malignant Pathogenesis

ISGs in Tumor and Other Cells
Decreased in constitutive expression
Increase correlates with improved prognosis
RNase L (HPC1) mutation and SNPs in IFN pathways increase prostate and colorectal cancer risk
Murine Tumor Development
Antibody to murine IFN hastens tumor emergence
IFNs decrease carcinogen-induced tumors
Role in T-Cell and Dendritic-Cell Maturation
Methylation Silencing of Genes for IFN Actions
ISGs (XAF1)
RASSF1A MAGE1 DAP kinase

IFN, Interferon; *ISG*, interferon-stimulated gene; *SNP*, single-nucleotide polymorphism.

Table 53-3 Pathogen-Associated Macromolecular Patterns (PAMPs) as Ligands and Toll-like Receptors (TLRs) Resulting in Innate and Acquired Immunity

Distinct Macromolecules of Pathogens
Recognition of ligands by TLR TLR1: diacyl lipopeptides TLR2/6: triacyl lipopeptides TLR3: dsRNA (poly I:C) TLR4: lipopolysaccharides TLR5: flagellin TLR7/8: ssRNA/imiquimod TLR9: CpG DNA Intracellular proteins activated RIG-I PKR 2′,5′-Oligoadenylate synthetase
Promote Activation of Innate and Acquired Immunity
Synthesis of induced proteins Interferons (type I) Cytokines and chemokines Effectors Dendritic cells, natural killer cells Amplification (IFN-γ, IL-2, IL-12) Antigen presentation

structures as well as target receptors. Based on these similarities and differences, there are three types of IFNs.[23,25-27] Type I IFNs include the multiple subtypes of the IFN-α family with multiple subtypes, IFN-β, IFN-ω, IFN-τ, IFN-κ, and IFN-ε.[23,25-28] The sole type II IFN is IFN-γ.[29] The more recently discovered type III IFNs are also known as IFN-λ or IL-28/29; there are three known members, λ1 (IL-29) and λ2/3 (IL-28 A/B) produced by mucosal epithelial cells.[26] The type III IFNs share structural homology and induction characteristics with type I IFNs, with cell lineage distribution of their unique receptors restricted to mucosal epithelial cells and plasmacytoid dendritic cells (pDCs).[26]

The genes for the human type I IFNs lack introns; genes, including those encoding 14 subspecies of IFN-α, are clustered on chromosome 9.[23,25,27] The corresponding 13 murine genes are clustered on chromosome 4. At the protein level, the human IFN-α subspecies share about 50% sequence identity; IFN-β is 22% and IFN-ω is 37% identical to the IFN-α. IFNs-α and IFN-β have 186 to 190 amino acids and contain a cleavable signal peptide resulting in secreted protein of 165 or 166 amino acids. Two Cys-Cys disulfide bonds are conserved among the proteins. Structure-function analysis has implicated the NH_2 termini of IFN-α molecules as important for their biological activity.[30] The gene encoding IFN-γ, located on human chromosome 12 (mouse chromosome 10), has three introns and encodes a protein of 146 amino acids; it functions as a dimer.[29] The structural homology of IFN-γ with type I IFNs is minimal. NK and T cells are the major source of IFN-γ. Type I IFNs are produced predominantly by dendritic cells but can be induced in all other cell types, including T cells, monocytes, fibroblasts, and epithelial cells.

The specific isoforms induced depend on both the cell type and the inducing agent. Virus or microbial infection through their gene products, such as dsRNA, ssRNA, dsDNA viral envelope proteins or prokaryotic cell wall constituents, triggers type I IFN synthesis.[31-34] These viral and microbial pathogen-associated molecular patterns (PAMPs) are recognized by specific membrane proteins called Toll-like receptors (TLRs) that initiate the signaling process culminating in IFN synthesis (Table 53-3). dsRNA, a common by-product of viral replication, is recognized by TLR-3, a protein present in endosomal membrane.[34] dsRNA can also be recognized by two cytosolic RNA-helicases, RIG-I and Mda5, both of which are upregulated by type I IFNs.[35] Viral single-stranded RNAs are recognized by TLR7 and TLR8

and viral DNA by TLR9, all of which are also present in endosomal membranes.[32,33]

Induction also occurs in bacterial PAMPs such as lipopolysaccharides and unmethylated CpG DNA.[33,36-38] Low molecular weight synthetic molecules that can be agonists of TLRs 7 to 9 and produce IFNs and ISGs have also been identified.[39-41] Different adaptor proteins connect these receptor proteins to specific protein kinases, such as TBK1 and IKK, which activate transcription factors including NFκB, IRF3, IRF7, and AP-1. For IFN-β gene induction, NFκB, the AP-1 complex composed of ATF2/c-jun and IRF1, and IRF3 or IRF7 are needed. These phosphorylated proteins form the enhanceosome complex at the IFN-β promoter.[42,43] Synthesis of different members of the IFN-α family and IFN-β can be temporally staggered. IFN-β induces IRF7 synthesis, which in turn induces transcription of IFN-α1 and other IFN-α genes.[31] Synthesis of IFNs and their actions are, therefore, intimately linked; inhibition of IFN signaling blocks robust production of IFN-α. IRF5 and IRF8 can also participate in IFN-α gene induction in specific situations.[44,45]

Interferon regulatory factors (IRFs), a family of nine transcription factors, have common DNA binding domains in their N-terminal domains. Their C-terminal domains, resembling the SMAD transcription factors in most IRFs, were first identified through the role of IRF1 in inducing IFN-β.[45-47] Although IRF3 and IRF7 are more important in inducing type I IFNs, IRF-1 and possibly IRF-5 may determine which species of IFNs are induced by TLR activation. IRF1 is expressed constitutively and in response to IFN-γ, as is IRF8, whereas IRF7 is mostly produced by type I IFNs. IRF7 amplifies the initial phosphorylated activation of constitutive IRF3 and other IRFs through TLRs, leading to additional cascades of production of IFNs by homo- and heterodimers of mostly IRF3 and IRF7 and potentially also induction of specific ISGs such as CXCL10.[45-47]

Polyethylene glycol (PEG)-conjugated IFNs have superior pharmaceutical properties compared with their unconjugated counterparts as a result of greater protection against proteolytic degradation, better solubility, and slower catabolism and excretion.[48-52] Because the half-life of IFN-α2 ranges from 4 to 8 hours and little is detected in serum 24 hours after administration, daily injections of IFN-α are required to achieve sustained clinical efficacy. Two PEG-IFNs have been developed and taken forward to clinical regulatory approval: a 40-kDa branched PEG conjugated to IFN-α2a and a 20-kDa PEG conjugated to IFN-α2b. Although they have a somewhat less specific activity, probably as a result of lower receptor affinity, preclinical and clinical studies have established a prolonged human pharmacological profile enabling effective weekly dose for PEG-IFNα2 as compared with daily IFN-α2.[48,49,52]

IFN Receptors and Signaling

IFNs bind to species-specific, heterodimeric cell surface transmembrane proteins that trigger signaling through their cytoplasmic domains.[23,27,53-55] The signaling pathways used by IFNs and other cytokines partially overlap, reflecting their common structural motifs with other members of the class II cytokine receptor family. To elicit a cellular response, the receptor for IFNs-α and IFN-β requires two subunits, IFNAR-1 and IFNAR-2. IFNs-α and IFN-β use and compete for the same receptor complex, although IFN-β interacts with the receptor heterodimer in a different way than does IFN-α2.[56,57] The ligand-specific anchor points induce a different conformational change in IFNAR1 that can result in greater activation of a subset of ISGs.[54,55,58] IFN-γ binds to a different heterodimeric receptor consisting of two subunits, IFNGR-1 and IFNGR-2.[27] The receptor for IFNs-λ consists of a unique subunit IFN-λR1 and a second subunit IL-10R2, the latter also a component of the receptor complex for IL-10, IL-22, and IL-26.[26]

Signals are transmitted to the promoters of ISGs and transcription induced.[23,59] Many of the same genes are also directly induced by dsRNA and viruses, which in part use different signaling pathways. Synthesis of some of the proteins that mediate IFN signaling, such as IRF7 and STAT1, are induced by IFNs as well, thus eliciting positive feedback responses. Proteins, such as constitutively expressed IRF2, a stable nuclear factor, downregulate both production of type I IFNs and synthesis of ISGs such as PKR, OAS, and IRF7.[31] Suppressors of cytokine signaling (SOCS) proteins that block IFN signaling are also induced so that, even in the continuous presence of IFNs, signaling is transient.[60,61] The critical role of both of these proteins in damping ISG antiviral and antitumor responses has been confirmed in IRF2$^{-/-}$ cells and SOCS1$^{-/-}$ mice.[62-64]

The critical event in triggering signaling is the ligand-initiated dimerization of the receptors that results in cascades of tyrosine phosphorylation. The ligand-activated cytoplasmic domains of receptors for IFNs signal by binding to JAKs (Janus kinases) and STATs (signal transducers and activators of transcription).[27,60,65,66] The cytoplasmic domains activate the two nonreceptor protein tyrosine kinases, Tyk2 and JAK 1, which phosphorylate STAT1 and STAT2 (Figure 53-1). Phosphorylated STATs form homomeric or heteromeric dimers that bind to specific cis-acting DNA sequences in the promoters of ISGs (see Figure 53-1). The principal transcription factor activated by type I IFNs is ISGF3, composed of activated STAT1, STAT2, and IRF9, the last specifically recognizing the ISRE (IFN-stimulated response element). Thus, for type I IFN signaling, seven proteins are essential: IFNAR1, IFNAR2, JAK1, Tyk2, STAT1, STAT2, and

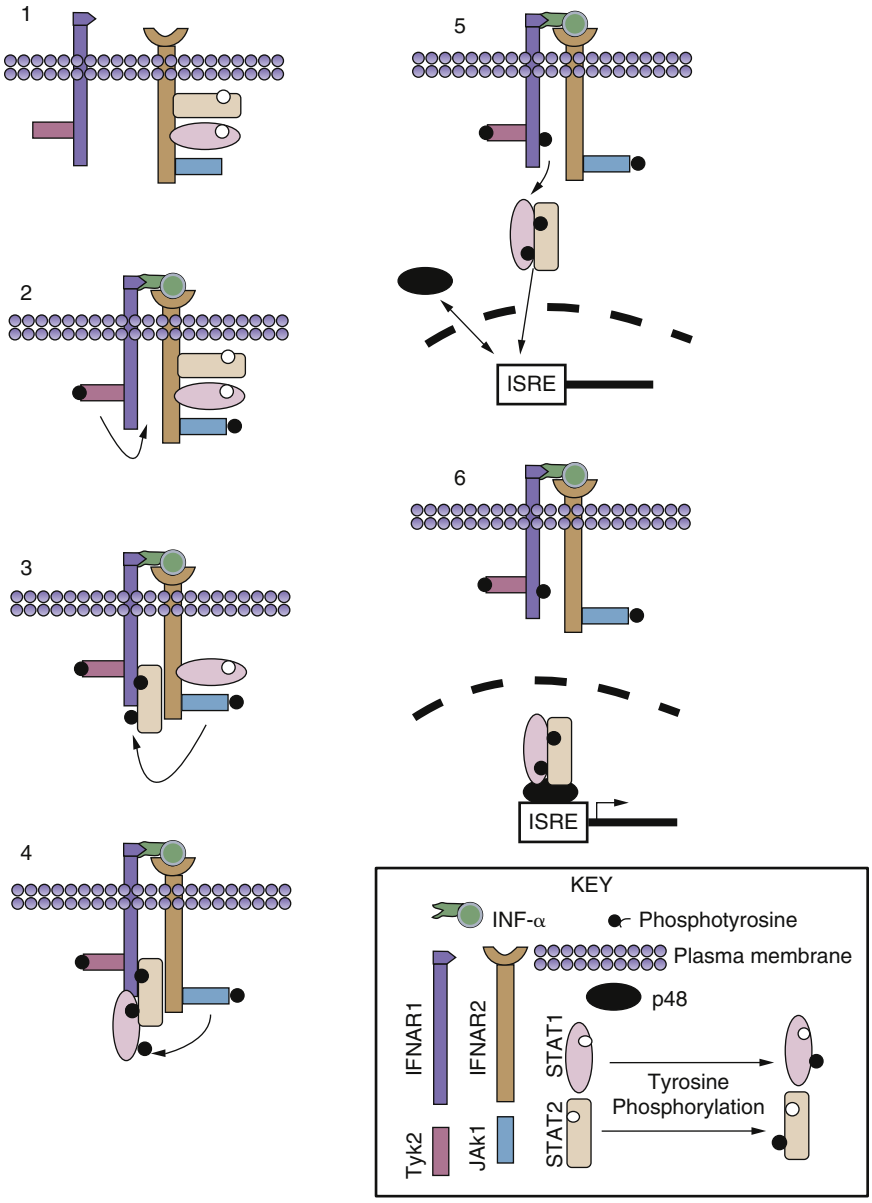


Figure 53-1 Signal transduction by IFNs-α and IFN-β (*1*) Receptor. (*2*) Ligand-induced aggregation of the receptor and phosphorylation of JAK1 and Tyk2. (*3*) Binding and phosphorylation of STAT2. (*4*) Binding of STAT1 to the tyrosine-phosphorylated STAT2 and its phosphorylation by JAK1. (*5*) Dissociation of STAT1-STAT2 heterodimer from the receptor and nuclear translocation. (*6*) Formation of ISGF3 complex and transcriptional activation. All proteins of ISGF3 complex make contacts with the bases in ISRE. *(Figure developed with thanks to D. Kalvokolanu.)*

IRF9. For full activation of STATs, further phosphorylation at specific serine residues is required as well. A dephosphorylation switch through the histone acetylation, leading to binding of phosphatase DCP45, suppressed signaling.[67]

Protein tyrosine phosphatases (PTPs) also have a regulatory role in suppressing signaling by IFNs in NK, T, and tumor cells. Inhibition of these can prolong signaling and potentiate antitumor activities of IFNs.[68-70] Loss of function of a phosphatase modifying STATs resulted in melanocytic neoplasms in *Drosophila*.[71] Inhibition of the PTP SHP-1 can be accomplished by inhibitors with potentiation of effects of IFNs.[70,72,73] SHP-2 silenced NK cells have elevated cytolytic activity with increased production of IFN-γ consistent with immune activation.[74] SHP-2 suppression increased STAT1 tyrosine 701 phosphorylation, increased promoter activity, and enhanced anti-melanoma effects of IFN-α2b for a human xenograft in mice.[70,75]

In the IFN-γ signaling pathway, the two receptor subunits IFNGR1 and IFNGR2, JAK1, JAK2, and STAT1 are required to activate the transcription complex, GAF, which binds to the gamma-activated site (GAS).[23,27] Additional Ser phosphorylation of STAT1 is required for the optimum function of GAF. Because the intracellular component of IFNγR2 at a site separate from the JAK2 binding domain can be inhibitory of apoptosis without ligand activation,[76] additional functions of this and possibly other components of IFN receptors may be identified.

IFN-λ proteins signal through a different receptor that is composed of a unique IFN-LR1 subunit and the IL-10R2 chain of IL-10, IL-22, and IL-26 receptors.[77] Like other type I IFNs, IFN-λ uses the proteins JAK1, Tyk2, STAT1, and

STAT2 for signaling; additional pathways are probably activated as well. Another major difference is in the expression profile of the receptors. Whereas IFNAR1 and IFNAR2 are ubiquitously expressed on cells of all lineages, expression of IFN-LR1 has a more limited distribution on epithelial and dendritic cells.[26,77]

In addition to the STAT pathways outlined earlier, IFNs can activate signaling pathways through other transcription factors.[23,66,78] For example, just like IFN-γ, type I IFNs can trigger STAT1-dimer formation and gene induction by the GAS. Furthermore unphosphorylated STAT1, itself induced potently by IFNs, can prolong and increase expression of ISGs initially induced by pSTAT1.[79] Signaling cross talk between STATs is illustrated by the effectiveness of an inhibitor of STAT3 in augmenting IFN-α activity for a murine melanoma.[80] This finding is in accord with those identifying roles of heterodimers of other STATs with STAT1.[78,81]

Although different subspecies of IFNs-α and IFN-β signal through the JAK-STAT pathway using the same receptor, signaling pathways triggered by them may not be identical. A mutant cell line lacking Tyk2 still responded to IFN-β and IFN-α8, but not to IFN-α1 and IFN-α2.[82] Preferential induction of the *BR1* gene by IFN-β also supports the notion of additional IFN-β-specific pathways.[83] The receptors, which are glycosylated in their extracellular domains, are both necessary and sufficient for activities of IFNs. Binding to IFNAR-1 accounts for the affinity and differential actions of IFNs-α and IFN-β.[54,84,85] IFNAR1 is at least in part degraded by an E3 ubiquitin ligase; decrease in activity of the ubiquitin ligase increases IFNAR1 and decreases tumorigenicity for a human melanoma xenograft.[86]

To add to the complexity of the signaling cascade, other STATs, such as STAT3 and STAT5, are activated by IFNs and can form homo- and heterodimers that influence transcription.[66,87] Indeed, in chronic lymphocytic leukemia (CLL) cells with high-risk molecular features, STAT3 was activated by IFNs, resulting in increased cell size and number.[88] Furthermore, STAT2 influences gene expression independently from its phosphorylation, with binding identified before IFN-α2 on many ISGs.[89] IFN type I suppressed genes were marked by pSTAT2 and by repressive histone methylation marks on H3K27me3 together with STAT1 binding.[89,90]

Ancillary pathways, activated by type I IFNs, are also triggered by additional protein kinases such as PI3K and p38 MAPK.[66,78] PI3K and p38 MAPK signaling pathways are activated by type I IFNs, and responses can be inhibited by low molecular weight inhibitors.[83,91,92] Purified $CD34^+$ cells with a mutation in JAK2, derived from patients with polycythemia vera, had increased apoptosis from pegylated IFN-α2a, an effect mediated by p38 MAPK.[93] Furthermore, MAPK activation may play an important role in translational synthesis of ISGs and thus antiproliferative effects.[94]

Thus, the global impact on gene induction by IFNs is influenced by many factors, including cell lineage, basal transcription binding and methylation of promoters, and exposure of the cells to other cytokines and secondary cascades of signaling generated by genes induced in the primary response.

IFN Pathways and Molecular Oncogenesis

Genotypic variation in signaling components in IFN pathways can be important in initiation and progression of malignancy. Oncogenes such as RAS and HPV E6E7 downregulate TLR signaling pathways,[95,96] possibly enhancing cell transformation. Several viral oncoproteins can block IFN signaling, often by interfering with the function of ISGs or ISGF-3.[97,98] IFN-κ with 30% homology to other type I proteins has been identified in epidermal keratinocytes and imparts cellular protection against viral infections in a species- and tissue-specific manner, using the same receptors and signaling pathways as other type I IFNs. It was suppressed by human papillomavirus type 16 in keratinocytes in vitro and in human cervical carcinoma biopsies, suggesting its role in innate immunity.[99]

The potential importance of TLR signaling in oncogenesis has been suggested by the identification of gain-of-function mutations in an adaptor protein (MYD88) for TLR signaling in a subset of aggressive diffuse B-cell lymphomas, particularly those of mucosa-associated lymphoid tissue (MALT).[100] A population-based control study of more than 4000 individuals identified single-nucleotide polymorphisms (SNPs) in IRFs, IFN-γ, and IFN-γR2 as influencing colorectal carcinoma risk and progression.[8] With an increasing number of variant genotypes, the risk of colorectal carcinoma and hazard of dying increased linearly.

The initiation and progression of carcinogen-induced bladder or hepatocellular carcinomas in murine models was inhibited by IFN-β or IFN-γ, respectively.[101,102] In the bladder carcinoma model, both IFNs and an inducer were effective; IFN-β completely prevented tumor formation. Gastric carcinogenesis from *Helicobacter pylori* and hepatocarcinogenesis from a chemical carcinogen in murine models was suppressed by IFN-γ through autophagy, p53 activation, and alterations in the tumor immune microenvironment.[102,103] In the hepatocellular carcinoma studies, although infiltration of immune effector cells and chronic inflammation was present, inhibition resulted from activation in hepatocytes of p53 tumor suppressor function.

Evidence suggesting a direct role for IFNs on tumor cell viability and progression through apoptosis or inhibition of proliferation has resulted from mouse tumor model systems. Treatment with human IFN-α or IFN-β of immunodeficient nude mice implanted with human tumor cells

effectively controlled tumor growth and promoted tumor cell apoptosis.[104,105] Alteration of tumor phenotype by reversal of epigenetic silencing of gene expression made an IFN-resistant mouse tumor sensitive.[106] Because mouse cells do not respond to human IFNs, these antitumor effects were clearly direct. Furthermore, progression of a RAS transformed cell line could be reversed both in vitro and in vivo by exogenous IFN-β with or without retinoic acid.[107] RAS can, however, result in activation of endogenous IFN-β that may contribute to the transformed cellular phenotype.[108]

Conversely, other murine studies highlight the importance of immune effector cells in antitumor effects of IFNs. Equivalent antitumor effectiveness of IFNs in vivo has been identified for syngeneic tumor cells sensitive or resistant to antiproliferative effects of IFNs in vitro.[109,110] Additional evidence supporting a role for host immune effector cell response to IFNs comes from studies in which mice implanted with a syngeneic leukemia, or with human prostate and HeLa tumor xenografts, received neutralizing antibody to murine IFN.[111] These mice, in the absence of exogenous IFN, had enhanced tumor growth and transplantability, suggesting that neutralization of endogenous IFN removes aspects of host defense to tumor.

Subsequent studies, using genetically altered mice, have more rigorously suggested the potential importance of immune cell regulation in the antitumor effects of type I IFNs. *STAT1* knockout mice implanted with IFN-responsive tumors and treated with exogenous IFN-α did not survive longer than control mice.[112] Furthermore, wild-type (*STAT1*$^{+/+}$) animals implanted with *STAT1*-null tumor cells were able to mount an effective antitumor response following IFNs suggesting the importance of host cells.[113] Additional studies of either IFN-γR– or STAT1-deficient mice identified the development of spontaneous tumors of diverse histologies or chemically induced fibrosarcomas at substantially greater frequency than in controls.[114,115] Type I IFNs, endogenously produced, were required to prevent primary carcinogen-induced and transplantable tumors.[116] In contrast to IFN-γ, for which tumor cells were important targets, host hematopoietic cells (NK cells, dendritic cells) were critical targets for IFNs-α and INF-β during the development of protective antitumor responses leading to tumor elimination.[116,117] Particularly critical for mediating effects of type I IFNs may be CD8α^{+} dendritic cells.[118,119]

Mice lacking IRF8 develop a disease similar to chronic myelogenous leukemia (CML). Overexpression of IRF8 in Bcr-Abl transformed cells inhibited leukemogenesis as well as resistance to the tyrosine kinase inhibitor imatinib; this correlated with reduced bcl-2 expression.[120] IRF4 inhibits myeloid progenitor cell growth and granulocytic differentiation in vitro, and its absence enhanced progression of the CML-like disease that occurs in IRF8$^{-/-}$ mice.[121] In chronic lymphocytic leukemia, heterozygous mutations in the DNA binding domain of IRF4, which both is constitutively expressed and can be induced through TLR ligation, have been identified in a small proportion of patients.[122] Other genetic perturbations in IRF4 have been identified in a subset of germinal center B-cell lymphomas (a translocation) and cutaneous carcinomas (a germline SNP).[123,124] Similarly, overexpression of IRF5 in breast carcinoma cells inhibited in vitro and in vivo cell growth and was identified as downregulated in human breast carcinomas.[125]

Over the past decade, paradoxes and additional complexities in antitumor actions of IFNs have been identified. STAT1 has generally been considered the upstream initiator of a prodeath and tumor-suppressive pathway. However, emerging evidence suggests that STAT1 activation and its induction by IFNs can be associated with resistance to DNA-damaging agents and may mediate more aggressive tumor growth.[79,126-128] STAT1 was induced by ionizing radiation or doxorubicin and can also be identified together with other ISGs as constitutively present in human tumors. Indeed, presence of a subset of ISGs in breast cancers was adversely prognostic for metastasis-free survival following adjuvant radiation and chemotherapy.[128] Specific ISGs, such as ISG15 and G1P3, have been identified as constitutively expressed in breast carcinomas and associated with anti-apoptotic effects and adverse clinical prognosis.[129-131] Chronic induction of ISGs may be detrimental, possibly through unphosphorylated STAT1 after an initial protective burst of ISGs in response to pSTAT1.[79] Understanding these adverse patterns of ISG expression on resistance to DNA damage and patient outcome may, over the next several years, further explain why IFNs are not more effective clinically.

Regardless of the cellular mechanisms augmented and suppressed by endogenous and exogenous IFNs, underlying the immunoregulatory, antiproliferative/prodeath, and antiangiogenic effects of IFNs are the ISGs, which are regulated at the level of transcription.[132] Although the function of many of these ISGs has begun to be understood, many were identified initially as differentially expressed mRNAs after the treatment of cells with IFNs or more recently on expression arrays. Although overlap in function certainly exists, actions relevant to antitumor effects of the most studied and potently induced protein products of ISGs are summarized next.

Mechanisms of Antitumor Action of Induced Genes

Underlying the antitumor actions of IFNs are the transcriptionally regulated ISGs.[132] Almost 2000 genes are transcriptionally regulated, mostly stimulated (more than 1500) but

some suppressed (about 300), with varying patterns of temporal expression over 24 hours after IFNs.[1,2,133] They are relatively equally divided between those predicted to be nuclear or cytoplasmic in functional location and include structural proteins, transcription factors, adaptors, enzymes, metabolic factors, and secreted proteins.[2] Fundamental discoveries concerning regulation of RNA stability and editing, translational protein synthesis, and protein transport and turnover have emerged from studies of these gene products. The proteins induced influence and mediate the pro-apoptotic, immune modulatory, and anti-angiogenic effects. Realizing that ISGs may have more than a single biological effect, some of the most potently induced (10× to 50× at the RNA level) have been related to these antitumor mechanisms of action.

Direct Antigrowth and Pro-apoptotic Effects

Regardless of direct or indirect causes of stasis or regression of tumors, antigrowth and apoptotic effects must result in the transformed cell. Thus activities of ISG products that may mediate or influence this action have been one key focus of assessment of molecular actions (Table 53-4). Unlike the rapid apoptosis induced by cytotoxics such as camptothecin, programmed cell death through the intrinsic cascade in response to IFNs is a later effect, requiring 48 to 72 hours. Underlying this latency is probably the requirement for synthesis of protein products of ISGs that occur over 24 to 48 hours (many identified by expression profiling).[1,134]

An ISG product, Apo2L/TRAIL (TNF-related apoptosis inducing ligand) mediates apoptosis from IFNs in myeloma, melanoma, T cells, B cells, and hepatoma and lymphoma cells.[135,136] IFN-β induced Apo2L/TRAIL in melanoma and other cells more potently than did IFN-α2.[135] Coculture of cells with IFN and neutralizing TRAIL antibodies or TRAIL decoy receptors can inhibit IFN-induced apoptosis. Apo2L/TRAIL induction in breast cancer cells by IFN and retinoic acid was mediated through IRF1 activation of the Apo2L/TRAIL promoter.[137] Combination with IFN and retinoic acid synergistically induced Apo2L/TRAIL with potentiated antitumor effects.[137]

Table 53-4 IFN-Regulated Proteins Contributing to Apoptosis

Apo2L/TRAIL	Fas ligand
IRF-1	XAF-1
PML	DAPK
RNase L	Protein kinase R
p56 family proteins	IFI-16, AIM-2 (p200 family proteins)
IHPK2	

Other ISG protein products are needed as well to sensitize cells to the effects of Apo2L/TRAIL. Pretreatment with IFNs, particularly IFN-β or IFN-γ, enhanced apoptosis from recombinant Apo2L/TRAIL or its receptor agonists, even in cells otherwise resistant to Apo2L/TRAIL.[135] Indeed, a gene signature dominated by ISGs predicted sensitivity to TRAIL for 95 human cancer cell lines.[138] ISGs, such as the p200 family, PML, Fas L, and XAF1,[139-146] among others, also have substantial effects on cell proliferation and viability. XAF1 was correlated with the ability of cells to respond to the pro-apoptotic effects of Apo2L/TRAIL.[142] Through interactions with p53 and the inhibitor of apoptosis, XIAP,[142,143] XAF1 may allow Apo2L/TRAIL to fully activate downstream caspases. Possible importance of XAF1 clinically has been suggested by finding that its constitutive expression, assessed by immunohistochemistry (IHC), in renal carcinomas was low in patients with shortened survival times.[147] Another ISG product, IRF1, suppressed another anti-apoptotic protein, survivin.[144]

The nuclear protein PML, which acts as a tumor suppressor, may be involved in IFN-induced Apo2L/TRAIL expression in myeloma cells.[140,145] Mice deficient in PML were largely resistant to IFN-induced apoptosis. Nuclear bodies (NB), multiprotein complexes in the nucleus associated with acute promyelocytic leukemia and acquired immunodeficiency syndrome (AIDS), are made up of the PML protein, sp100, sp140, sp110, and the exonuclease ISG-20, all of which are ISG products.[145] In contrast to the pro-apoptotic actions of products of many ISGs, G1P3 (ISG6-16) inhibited apoptosis both in gastric and breast carcinomas and myeloma.[129,148,149]

In CML and multiple myeloma cells, induction by IFN-α of the death receptor Fas (CD95) resulted in apoptosis through activation of caspase-8/FLICE.[150] Intralesional administration of IFN-α into basal cell carcinomas increased Fas expression and led to regression.[151] Similarly, IFN-γ increased susceptibility of melanoma cells to apoptosis by Fas activators and breast carcinoma cells to doxorubicin.[152,153] Doxorubicin also induced STAT1.[152] Among other genes induced in an ovarian carcinoma xenograft regressing after paclitaxel were ISGs (G1P3, IFI16, IFI27, IFITM1, and ISG15).[154]

Treatment of cells with IFNs results in sustained activation of the latent endoribonuclease RNase L, which degrades ssRNA and triggers a mitochondrial pathway of apoptosis that eliminates virus-infected cells.[4,155,156] Genetic disruption of RNase L impairs this apoptotic response, which has raised interest in the possibility that such mutations might also contribute to malignancy (Figure 53-2).[157,158] In this regard, the hereditary prostate cancer 1 (*HPC1*) gene maps to the RNase L gene (*RNASEL*) and is implicated in controlling apoptosis of prostate cancer cells.[3]

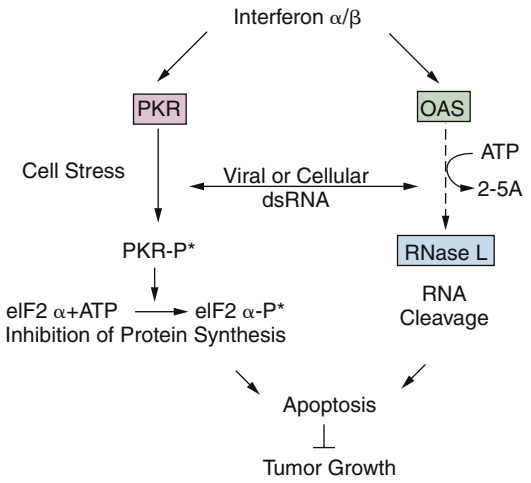


Figure 53-2 Interferon-regulated activation of 2-5A and PKR pathways and their mechanisms of action.

In a large, controlled sibling-pair study, an RNase L variant (R462Q) with decreased enzymatic activity was associated in up to 13% of unselected prostate cancer cases.[3] However, although case-controlled genetic and epidemiologic studies support the involvement of *RNASEL* (and notably the R462Q variant) in prostate cancer etiology,[3,5,6] others do not,[159,160] suggesting that either population differences or environmental factors such as infections may modulate the impact of *RNASEL* on prostatic carcinogenesis. A novel xenotropic murine retrovirus (XMRV) was identified in the prostate tumor-bearing tissues, almost exclusively in men with the homozygous, reduced activity variant of RNase L (R462Q).[161] Other findings suggest a wider tumor suppressor role of RNase L in additional malignancies.[162,163]

Activation of RNase L is the only well-established function of the ISG products 2′,5′-oligoadenylate synthetase (OAS).[164] The OAS-RNase L system (see Figure 53-2) in humans has four functional *OAS* genes (*OAS1-4*), resulting in 8 to 10 OAS isoforms because of alternative mRNA splicing.[164] When stimulated by double-stranded RNA (dsRNA), OAS proteins produce from ATP a series of short 5′-phosphorylated, 2′,5′-linked oligoadenylates collectively referred to as 2-5A [$p_x5'A(2'p5'A)_n$; x = 1-3; n ≥ 2]. Because dsRNA is a frequent viral PAMP, 2-5A often accumulates in IFN-treated and virally infected cells.[165] In contrast, OAS1 mRNA and other ISGs were reduced in prostatic malignancies.[166] OAS2 has been specifically associated with the fatigue and depression associated with chronic administration of IFN-α2 to patients.[167]

Another ISG, protein kinase R (PKR), mediates signal transduction, apoptotic, and tumorigenic responses (see Figure 53-2).[168,169] PKR, an IFN-inducible, dsRNA-dependent serine/threonine protein kinase that inhibits protein synthesis initiation, is activated by dsRNA, dimerization, and autophosphorylation.[170,171] PKR is a 551-amino-acid protein in which the N-terminal region contains two dsRNA binding motifs, whereas the catalytic domain is present in the C-terminal half.[172,173] In addition, a cellular protein (PACT) can directly activate PKR in the absence of dsRNA.[174] PKR is a p53-regulated gene with an influence on p53-mediated inhibition of translation, apoptosis, and tumor growth.[175] Phosphorylation by PKR of the protein synthesis initiation factor eIF-2α caused an inactive complex to form between eIF2-GDP and the recycling factor, eIF2B. These events result in global inhibition of rates of protein synthesis both viral and cellular; indeed, many viruses evade the IFN response by inhibiting PKR function.[97]

Mouse fibroblasts lacking PKR are resistant to apoptosis induced by TNF, dsRNA, or lipopolysaccharide.[169] Phosphorylation of eIF2α by PKR is required for the apoptotic responses to dsRNA, TNF-α, or serum deprivation.[176] Implantation into mice of NIH3T3 fibroblasts expressing a catalytically inactive, dominant negative PKR led to tumorigenesis.[177] Suggesting further its key role in mediating the growth-suppressive effects of IFN-γ for $CD34^+$ myeloid precursors was the finding that pharmacologic or siRNA-mediated ablation of PKR increased hematopoietic colony formation.[178] Deficiencies in PKR activity, but not protein levels, have been identified in B-cell chronic lymphocytic leukemia.[179] Because PKR is a key point of regulation in the protein synthetic machinery, is induced by p53 in addition to IFNs, and can cause apoptosis, strategies for selectively activating PKR have been explored. Like RNase L, PKR exists in a latent, inactive state, and thus drugs that act as molecular switches for these proteins might have potent antitumor and/or antiviral effects.[180]

Adenosine deaminases acting on RNA (ADAR), another family of ISGs, through their effects on dsRNAs, can modulate cellular effects of IFNs.[181] Through their RNA editing activity, ADARs can affect translational gene expression via regulation by miRNAs. In addition, ADAR 1 is essential for hematopoietic stem cell survival and can have anti-apoptotic effects, possibly through modification of dsRNA essential for actions of OAS and PKR.[182,183]

The ISGp56 gene (also known as IFIT1), as are other ISGs discussed in this chapter, is strongly transcriptionally induced (50× to 100×) by type I IFNs.[184] These proteins (including related p54, p56, and p60) contain multiple tetratricopeptide repeat (TPR) motifs that mediate protein-protein interactions.[184] Although the human p56 and the mouse p56 proteins have only 50% sequence identity, their six TPR motifs are positioned very similarly along the linear sequences of their amino acids. A major property of the p56 and p54 proteins is inhibition of initiation of protein synthesis.[185,186] This inhibition is mediated by binding to specific subunits of the translation initiation factor eIF3; human p56 binds to the e subunit, whereas p54 binds to both subunits.

Binding of these proteins to the e subunit blocks the ability of eIF3 to stabilize the ternary complex of eIF2, GTP, and Met tRNA, blocking CAP-dependent translation initiation. These alterations in protein synthesis may underlie the pro-apoptotic effects of IFIT2 through caspase 3 that can occur without IFN stimulation.[187] The subunit of eIF3 to which p56 bound was identical to *Int-6*, a gene whose disruption by the integration of the mouse mammary tumor virus gene caused mouse mammary carcinoma.[188] The cellular function of the eIF3e/Int-6 protein in this context could be different from translation initiation because the protein was present not only in the cytoplasm as a component of eIF3 but also in the nucleus.

The p200 family of ISG protein products, IFI-16, myeloid cell nuclear differentiation antigen (MNDA), and absent in melanoma 2 (AIM2), share a common 90-amino-acid, N-terminal pyrin domain and a partially conserved 200-amino-acid C-terminal domain important for protein-protein interaction.[189] p200 family members have a significant role in cellular growth regulation and differentiation.[141,189-194] They influence function of the tumor suppressor genes Rb and p53 and the transcription factor E2F. Inhibition of proliferation by these p200 gene products has resulted in loss of the transformed phenotype and decreased tumor formation in mouse models.[189] In an IFI16-null human tumor cell line, retroviral restoration of IFI16 expression reduced tumorigenesis significantly.[195] Similarly, AIM2 suppressed breast carcinoma and colorectal cell growth.[196,197] In human prostate carcinoma cells, overexpression of IFI16 resulted in a senescence-like phenotype with reduction in S-phase–specific cells and inhibition of colony formation.[198] Studies such as these have established the p200 family as among the most active in growth suppression and most pro-apoptotic of ISGs.[189]

Identified initially as an IFN-γ induced pro-apoptotic gene, death-associated protein kinases (DAPKs) are serine threonine kinases with ankyrin and death domains that induce caspase-independent cell death.[199] Reduced expression correlated with malignant progression, whereas restoration of expression led to apoptosis of mouse lung tumors.[200] Expression of DAPK can be suppressed in human malignancies, at least in part by epigenetic silencing.[201] IFN-γ resulted in induction of a novel growth suppressive pathway through the transcription factor C/EBPβ.[202] IFN-γ stabilized DAPKs through inhibiting its ubiquitination, thus identifying another mechanism by which IFNs promote apoptosis and autophagy.[203]

Immune Effects

Amplification of innate and specific immune responses results from both type I and type II IFNs.[20,57] The antitumor activity of IFNs in vivo is mediated by activation of immune effector cells, increased antigen processing, and enhanced immunogenicity of tumor cells (Table 53-5). Plasmacytoid dendritic cells have been identified to secrete high levels of IFNs-α and IFN-β in response to stimuli, have been found associated with the T-cell zone of lymphatic tissue, and have been identified in tumors of various types.[16,18,19,204-206] Production of type I IFNs is a process partially regulated by osteopontin and furthers dendritic cell maturation and induction of T cells into a Th1 pathway.

Table 53-5 IFN-Regulated Proteins Contributing to Immune Response

MHC Class I	MHC Class II
LMP-2, LMP-7	TAP
CEA	TAG-72
CCL chemokines	CXC and CXCL chemokines
Phospholipid scramblase	ISG-15

IFNs can stimulate activity of cytotoxic and helper T cells, NK cells, macrophages, and dendritic cells. Cytotoxic T-cell expansion was promoted by IFN-γ in part through upregulation of IL-2.[207] Activation of NK cells and macrophages has been identified both in vitro and in vivo.[208] Furthermore, IFNs induced expression of Apo2L/TRAIL on immune effector cell surfaces, sensitizing tumor cells to T-cell and NK-cell–mediated cytotoxicity.[209,210] Type I IFNs can also suppress T-cell function through inhibiting secretion of IL-17.[211,212] Type I IFNs can induce IL-10 in T cells, thus dampening both innate and adaptive immune responses.[213] Reduced responsiveness in T cells to IFN-α and IFN-γ also resulted from myeloid-derived suppressor cells through reduction in p-STAT1.[214]

In addition to both stimulating immune effector cells and constraining inhibitory immune subpopulations, IFNs upregulated major histocompatibility complex (MHC) antigens facilitating activation of $CD8^+$ cytotoxic T cells.[215-217] IFNs increased transcription of MHC class I genes but also coordinately induced expression of additional proteins required for the surface expression of the mature MHC class I complex. As nascent class I molecules are synthesized, they associate with β2-microglobulin, also an ISG, in the endoplasmic reticulum for transport to the cell surface. Augmentation of HLA class I–associated β2-microglobulin has been identified in patients after IFN-α2 or IFN-β.[218,219] The effects of IFNs in increasing HLA class II genes may be in part mediated by the HIN-200 family member AIM2, suggesting a novel role for this ISG in specific immune responses.[220]

Generation of antigenic peptides for loading onto class I molecules occurs in the proteasome. The three subunits, low molecular weight protein-2 (LMP-2), LMP-7, and LMP-10,

that make up the proteasome and the cytoplasmic transporter for processing (TAP) are all ISGs, can also be induced by IRF7, and are suppressed in some malignancies.[221-224] TAP transports the processed peptides from cytosol to the ER for loading onto class I molecules. The MHC class II transactivator factor (CIITA), the master regulator of MHC class II expression, is induced by IFN-γ.[57] In addition, three cathepsins, thought to be partly responsible for peptide antigen processing and loading onto MHC class II proteins, were upregulated by IFN-γ.[225] In addition to IFN-γ, type I IFNs can also drive MHC class II expression onto the plasma membrane and also maintained intracellular vacuolar expression for sustained dendritic-cell presentation of antigens.[226]

Through their immune effector functions, IFNs type I and IFN-γ can have an amplifying role for other immunomodulatory therapeutics. IFNs induced the tumor-associated antigens carcinoembryonic antigen and TAG-72 on the surface of tumor cells both in vitro and in vivo.[227] Type I IFNs induced by a vaccine protein linked to a TLR7/8 agonist markedly increased dendritic-cell antigen uptake.[228] This increase could be beneficial for either innate or adoptive immunotherapies targeting these tumor antigens, as suggested by studies combining IFN-α with a CEA poxvirus vaccine.[229] Offering the potential for use with targeted monoclonal antibodies directed at tumor proteins was the finding of the essential role of both type I IFNs and IFN-γ in mediating effectiveness of a monoclonal antibody binding to HER2/ErbB-2 in a murine breast carcinoma model.[230] This effect required TLR signaling through MyD88 and IFN-γ producing CD8 T cells but was independent of perforin, fas ligand, or IL17R signaling.

IFNs have increased expression of a number of chemokines that function as chemoattractants both in vitro and in patients.[231,232] IFN-induced chemokines include CCL5 (RANTES), CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α), CXCL9 (MIG), and CXCL11(I-TAC). STAT1 induction of chemokines CXCL9, CXCL10, CXCL11, and CXCL16 controlled recruitment of protective, antigen-specific Th1 cells into peripheral tissues.[233] These ISGs and IRF-induced proteins are chemoattractive to both lymphocytes and monocytes and play a crucial role in recruiting these cells into tissues.[234]

Other ISG products, such as phospholipid scramblase 1 (PLSCR1), may play a role in providing macrophages with a signal for engulfment after tumor cell apoptosis.[235-237] PLSCR1 may increase expression of a subset of ISGs influencing apoptosis and other effects of IFNs.[235-237] Ovarian cancer cells overexpressing PLSCR1 grew more slowly in nude mice compared to vector-transfected cells, and resultant tumors were infiltrated by neutrophils and macrophages. Thus by inducing PLSCR1, IFNs may facilitate tumor cell phagocytosis by macrophages.

ISG-15, a secreted protein induced by IFNs-α and IFN-β, induced IFN-γ synthesis by T cells and proliferation of NK and lymphokine-activated killer (LAK) cells.[238] A genetic defect in ISG15 secretion resulted in impaired production of IFN-γ by NK and T cells and impaired protective immunity to mycobacteria.[239] Four of the top 36 expressed genes in an expression array of melanoma cells (ISG 15, USP18, UBE1L, UBE2L6) constitute part of a cascade of ubiquitin-related ISGs that influence both innate immunity for malignant cells and cellular signaling. These can conjugate intracellular proteins to influence action and extracellular cell function of IFNs.[240-244] Melanoma cells producing high levels of ISG-15 were those able to induce e-cadherin expression on dendritic cells.[241] Conjugation of ISG15 to components of the IFN-signaling pathway (STAT1 and JAK1) and other signaling pathways (ERK 1, phospholipase C, and heat shock proteins) suggest that targeted regulation of ISG 15 family molecules could have profound effects not only on tumor cells but also on host response.[244]

Consistent with the concept that ISG15-ylation of proteins is analogous to ubiquitination, most target identified proteins have decreased function with ISG15 conjugation.[244] However, an siRNA to the ISG USP18, the ISG that deconjugates ISG15 from target proteins, increased expression of TRAIL through its increased transcription and thus promoted apoptosis.[245] Consistent with this is the finding that immunization against ISG15 resulted in CD8 T-cell–mediated reductions in murine breast carcinoma growth.[246] Complexities in understanding the functional effects of the ubiquitin-like ISGs may be explained by ISGylation and deISGylation stabilizing or altering binding of a target protein to its typical interaction partner at the protein-protein or protein-RNA interface.[244,247]

Inhibition of Malignant Cell Motility and Invasion

An essential part of the malignant process is the motility of transformed cells and their invasion of normal tissues, a process inhibited by several of the highly induced ISGs. One of the genes highly induced by IFNs-α and IFN-β in most tissues is *MX1*. It encodes a GTPase MxA that inhibited motility and invasiveness of prostate carcinoma and melanoma in both in vitro and in vivo assessments.[248] IFIT2 (p54) inhibited migration of oral squamous carcinomas in vitro, possibly through interaction with cytokeratins.[249] Another ISG product, schlafen 5, when suppressed, increased melanoma cell invasiveness into three-dimensional collagen.[250] Because schlafen 5 was often suppressed in melanoma, it may contribute to antimelanoma effects of IFN-α2.[250]

Though less widely studied for its biological effects than some others, the products of the ISGs of the IFITM family are transmembrane proteins (IFITM1, 9-27; IFITM2, 1-8) of wide phylogenetic distribution, expression in most tissues, and lower molecular weight (approximately 14 to 17 kDa).[251] IFITM1 associates with cell surface CD81 in many human cells types, and the complex is involved in β1 integrin adhesion to extracellular matrix proteins.[252] It also interacts with the CD19/CR2 signal complex in lymphocytes and antibodies to IFITM1 and resulted in homotypic adhesion of T cells.[253,254] The antibody to IFITM1 has antiproliferative effects for leukemic B cells; thus IFITM1 contributes to growth control in lymphocytes.[255,256] Upregulation of IFITM1 and IFITM3 has been identified in more invasive breast, head and neck, and gastric carcinomas with promotion of invasiveness in vitro.[257,258] Also suggesting a role in homotypic adhesion and greater invasiveness was the lower level of IFITM1 and IFITM3 in metastatic melanomas.[259]

Angiogenesis Inhibition

IFNs inhibit angiogenesis both by altering the stimulus of tumor cells and by inhibiting endothelial cells through increase and decrease of proteins mediating the angiogenic process (Table 53-6). Following IFNs, vessels underwent coagulation necrosis.[260] Inhibition of angiogenesis by IFNs occurred before antiproliferative effects on tumor cells and has been identified in vivo within 24 hours of tumor-cell inoculation.[261] IFN-sensitive and IFN-resistant bladder carcinoma cells in mice had reductions in tumor cell growth after IFN-α in IFN-sensitive cells by directly regulating expression of the angiogenic cytokine basic fibroblast growth factor (bFGF).[262] Suppression of bFGF correlated with reduced vascularization and tumor growth.[262] Knockout studies confirmed signaling through STAT1 as necessary for reduction by IFNs of bFGF signaling. Compared with wild-type mice, IFNAR receptor knockout mice have increased angiogenesis and tumorigenesis.[263]

In addition to their action on bFGF, IFNs have inhibited angiogenesis by acting on other angiogenesis mediators. IFNs inhibited VEGF mRNA and protein expression in neuroendocrine tumors by regulating VEGF promoter activity.[264] Effects on VEGF promoters may in part be mediated by a reduction in one of its promoters, HIF-1α, that was reduced by IFN-γ.[265] Contributing to reductions in VEGFA by IFN-γ may be a novel conformational change in the VEGF-A RNA 3′ untranslated region to reduce VEGF-A translation through integration of hypoxia and STAT signaling.[266] IL-8, a mediator of angiogenesis, was inhibited in vitro and in vivo by IFN-α2b and IFN-β.[267,268] Other angiogenesis inhibitory members of the chemokine family that lack the ELR binding motif, CXCL9, CXCL10, and CXCL11, are also IFN-stimulated genes.[269,270]

Table 53-6 IFN-Regulated Proteins Contributing to Suppressive Events in the Microenvironment

bFGF decrease	VEGF decrease
GBP1	IL-8 decrease
CXCL 9, CXCL 10, CXCL 11	Tryptophanyl-tRNA synthetase
MXA	schlafen 5
TFITM family	

Guanylate binding proteins (GBPs), a family seven human GTPases of the dynamin superfamily potently induced by IFNs, modulated proliferation and spreading of endothelial cells in vitro.[271] In endothelial cells, hGBP1 functioned as an inflammatory response factor, inhibiting endothelial cell proliferation and angiogenesis through matrix metalloproteinases.[272] hGBP1 also inhibited proliferation of endothelial cells stimulated by VEGF or bFGF through its C-terminal alpha helices.[273] In contrast to endothelial cell inhibition by hGBP1 was its increase in malignant glioma cells through EGF receptor signaling resulting in increased matrix metalloproteinase 1 and glioma cell invasion.[274] Whether this latter effect is transformed-cell or tissue-type dependent remains to be determined; it does, however, emphasize that induction and action of ISGs can occur through unique and undiscovered pathways that are independent of IFNs as ligands. Thus, induction of ISGs such as GBP1 and IFI16 that function as angiostatic inhibitors, coupled with downregulation of other factors, may contribute to inhibition of angiogenesis by IFNs.[271,275,276]

Endogenous type I IFN signaling may therefore play a role as a negative regulator of angiogenesis, keeping the "angiogenic switch" in the off position. Indeed, mice lacking IFN-β (IFN-$\beta^{-/-}$) had faster growing melanomas and sarcomas with better developed blood vessels than did control mice.[277] Tumors from these mice had enhanced infiltration by neutrophilic cell population with phenotypic markers of myeloid-derived suppressor cells (MDSCs) and increased expression of VEGF and matrix metalloproteinase 9. These data suggest that endogenous IFN-β may play an important role in regulating tumor-induced angiogenesis. Further supporting this was the finding that in vitro treatment of tumor-infiltrating neutrophils with low levels of IFN-β reduced expression of pro-angiogenic factors to normal levels.[277] In this and other preclinical mechanistic studies, IFNs inhibited tumor vascularization and modulated expression of pro-angiogenic and anti-angiogenic genes at doses well within those that are therapeutically achievable.[278] Consistent with an important role for angiogenesis inhibition in the antitumor effects of IFNs is that, clinically, IFN-α2b has proven

effective in the treatment of infantile hemangiomas, hemangioblastomas, giant-cell tumor of the mandible, and Kaposi's sarcoma.[279-281]

Antitumor Effects in Humans

Because of their clinical effectiveness in reducing tumor cell mass, limiting virus replication, controlling disease symptoms, and prolonging survival, IFNs are now licensed in more than 50 countries for treatment of viral, malignant, and immune disorders. Global market sales approximated $5 billion in 2010 and are expected to continue to increase to $10 billion by 2015. IFNs as single agents can induce clinical regression in almost a dozen hematopoietic malignancies or solid tumors.[23] In CML, melanoma, renal cell carcinoma, bladder carcinoma, Kaposi's sarcoma, hairy-cell leukemia, lymphomas, myeloma, polycythemia vera, locally advanced basal cell carcinoma, and essential thrombocythemia, IFNs have had therapeutic value.

For example, use of IFN-α2 for hairy-cell leukemia and for CML resulted in a gradual decrease in bone marrow infiltration with malignant cells as well as a normalization of peripheral hematologic parameters.[282-286] In CML, in addition to reduction in leukemic cell mass, a decrease resulted in cells with the abnormal, activated bcr-abl kinase. The median survival for all patients with CML treated with IFN-α2 was extended to approximately 6 years, with over 90% of those with complete cytogenetic response in remission at 10 years. Frequency of cytogenetic response and survival were further enhanced by adding cytosine arabinoside to IFN-α2. The survival advantage for IFN-α2 has now been exceeded by the substantial effectiveness of the targeted tyrosine kinase inhibitors such as imatinib and nilotinib. However, the hematologic effectiveness and tolerability of IFN-α2 and pegylated IFNα2 have resulted in continued evaluation in combination with imatinib with higher and longer duration molecular responses, sustained immunologic effects, and possible improvements in survival.[287-290] Aberrant JAK2 expression, part of a protein complex driven by the Bcr-Abl translocation in CML, could be a target.[291] Because the activity of IFN-α2 in CML has not been elucidated on a molecular basis, tyk2 and JAK1 stimulation by IFN-α2 could be providing a lateral, suppressive shift in from JAK2 activity with subsequent inhibition of proliferation. Furthermore, with its clinical effectiveness and tolerance, pegylated IFN-α2 continues to be evaluated and used for other myeloproliferative disorders such as essential thrombocythemia and polycythemia vera, with beneficial clinical results confirmed by reduction in cells with pathogenic mutations in JAK2(V617F).[292]

Curative elimination of metastatic malignancies can result when systemic therapies are given to eliminate micrometastases in patients at highest risk for recurrence after surgical removal of a primary. Effectiveness of IFNs as adjuvant therapy after surgery of murine tumors has been demonstrated and is consistent with pioneering clinical studies of IFNs in therapy for osteosarcoma involved this approach.[293,294] IFN-α2 enhanced clinical benefit of surgery and resulted in effects equivalent to effective chemotherapy. To more rigorously define the role of pegylated IFN-α2 after surgery and chemotherapy for primary osteosarcoma, a randomized international trial involving cooperative groups in Europe and the United States is ongoing.

Elimination of micrometastases was the basis for evaluation of IFN-α2b for patients at high risk for recurrence of melanoma—those patients marked by deeply invasive primaries or nodal metastases. The initial positive findings have been largely further validated by subsequent trials of high-dose IFN-α2b for 1 year and by meta-analyses.[295,296] A multi-institutional randomized trial evaluated pegylated IFN-α2 in more than 1200 patients with stage III melanoma for a period of 5 years with dose adjusted for the side effect of fatigue to maintain full activity level.[297] Overall survival was not significantly different in the two groups, although the IFN-treated patients had a significant improvement in relapse-free survival (RFS) ($P = .01$), with subgroup analyses identifying the greatest improvement in RFS in patients with microscopic nodal disease ($P = .02$).[297] Subsequent analysis of this study in a combined database identified an improvement in both disease-free survival and overall survival in patients with ulcerated primaries, this group possibly reflecting those with the most marked invasiveness or vascularity.[297]

Adjuvant use of IFN-α2 has also proved effective after resection and/or ablation for hepatitis C virus–associated hepatocellular carcinoma in both individual Phase II and randomized controlled Phase III trials and a meta-analysis. In the meta-analysis, regardless of viral load, highly beneficial effects on both reduction in recurrence frequency and overall survival were identified.[298] Building on the therapeutic activity of IFNs in Phase II trials in metastatic renal cell carcinoma (RCC),[299] resection of a primary followed by IFN-α2 extended patient survival in each trial cohort and in a combined analysis.[300] However, addition of the antiangiogenic bevacizumab to IFNs has more recently failed to markedly improve outcomes in metastatic RCC.[301] Furthermore, as in CML, the orally active, targeted kinase inhibitors such as sunitinib and axitinib have changed the natural history of RCC carcinoma, extending survival in metastatic disease more than the injectable IFN-α2.[302] In follicular lymphomas in combination with chemotherapy, even relatively limited amounts of IFN-α2 can prolong survival.[303] Curative

effects with topical use have been seen in both ocular squamous neoplasia and superficial carcinoma of the bladder. The former, treated with ophthalmologic drops of IFN-α2b, results in complete clinical regression in more than 80% of treated eyes—even those with T3 tumors.[304]

IFN-γ has had only limited clinical utility in melanoma, renal, colorectal carcinomas, and other metastatic malignancies as a single agent.[133,305,306] It has also been assessed in resected stage I and stage II primaries in a randomized study of patients.[307] Compared with untreated patients, no improvements in outcome resulted. More recent insights have begun to offer some understanding of this unexpected lack of clinical effectiveness of IFN-γ. Murine melanomas after IFN-γ, for example, evaded CD8 T-cell cytotoxicity by producing large amounts of tumor antigen, noncognate MHC class I molecules on melanoma cells, limiting T-cell activation and effector function.[308] Immune evasion and progression of a UVB-induced melanoma in mice was facilitated by IFN-γ through macrophage trafficking by induced chemokines.[309] Furthermore, TLR signaling or IFN-γ can induce an immunosuppressive T-cell ligand, PD-L1, in tumor cells, induction by IFN-γ occurring possibly through a MAPK signaling pathway.[310,311] This occurred in a time- and dose-dependent manner with suppression of CD8 T-cell cytotoxicity.[310,311] It is thus conceivable that on balance, the potent immunologic effects of IFN-γ may be detrimental rather than beneficial in the tumor microenvironment.

As inducers of IFNs, agonists for TLR7-9, imiquimod and the phosphothioate oligoribonucelotide CpG7909, have immunomodulatory effects.[312,313] Imiquimod has proven effective topically both for venereal warts caused by papillomaviruses and for basal-cell carcinomas.[314,315] Newer TLR9 agonists have been combined with rituximab and a CEA vaccine.[316,317]

Perspective

Both as a product of nascent biotechnology companies and as one of the several advances in modulating the immune system to treat cancer, introduction and clinical use of IFNs has been one of the major advances in oncology over the past three decades. The 1980s saw the clinical introduction of these high purified pharmaceuticals as the first products of biotechnology for the treatment of cancer. The 1990s were marked by an expansion in clinical use and a clearer understanding of the molecular events that influence the biologic actions. During these two decades, IFNs were understood as fundamental cellular defense mechanisms against viral infections and cancer and thus critically important to the health of animals and humans. Through the action of specific ISGs reviewed in this chapter, IFNs were identified as the principal cytokine that blocks viral replication, important in resistance not only to acute but also chronic human infections, such as those with oncogenic hepatitis C virus and papillomaviruses. Because biologic effects of IFNs were identified as mediated primarily through STAT1 signaling and its induction and the action of other induced ISGs, attention over these final 20 years of the 20th century shifted to understanding how insights into the genes reviewed in this chapter might lead to more efficacious cancer and new antiviral therapeutics.

The first decade of the 21st century has been marked by an application of this knowledge to further understand mechanisms of action of IFNs. Complexities in STAT activation, signaling, and regulation, possibly in some instances detrimental to endogenous and exogenous antitumor effects, have been identified.[79,128] As part of the innate immune response and immune surveillance mediated through TLR activation, new insights into the essential role of both type I and type II IFN signaling have, however, been identified.[20-22] Tumor cells that are not eliminated are kept dormant by a suppressive host response, both as the result of essential type I IFNs and IFN-γ.[20,21] Consistent with these murine observations, suppression or downregulation of STAT1-induced genes in tumor-infiltrated T cells and in peripheral blood of patients with metastatic melanoma has been clinically associated with worse outcome.[318,319]

Many important questions thus remain unanswered. What roles do endogenous triggering of TLRs play in antitumor responses? What specific roles do the multiple isoforms of IFN-α have in host defenses? How do STAT proteins regulate apoptosis, cell growth, immunity, and cytotoxic drug resistance? What are the functions of the many still-uncharacterized ISGs? Which of the ISGs are most important for the antitumor effects—and through what cellular mechanisms: immune augmentation, cell stasis and death, inhibition of angiogenesis? What mechanisms cause the difficult spectrum of clinical side effects of fatigue and anorexia? What causes resistance to IFNs in cancer? Can effective oral systemic inducers of IFNs or ISGs be identified? Answers to these questions will continue to stimulate future advances.

Acknowledgment

This chapter is revised and updated from the same chapter in the prior edition of *Molecular Basis of Cancer*. That chapter was written by the author together with R. Silverman and G. Sen of the Cleveland Clinic, whose prior contributions provided important elements of this update.

Key References

2. Hertzog P, Forster S, Samarajiwa S. Systems biology of interferon responses. *J Interferon Cytokine Res*. 2011;31:5-11.
21. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.
23. Borden EC, Sen GC, Uze G, et al. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov*. 2007;6:975-990.
24. Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A*. 1994;91:1198-1205.
27. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*. 2004;202:8-32.
31. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity*. 2006;25:349-360.
32. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373-384.
57. Trinchieri G. Type I interferon: friend or foe? *J Exp Med*. 2010;207:2053-2063.

References

1. Der SD, Zhou A, Williams BR, Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A*. 1998;95:15623-15628.
2. Hertzog P, Forster S, Samarajiwa S. Systems biology of interferon responses. *J Interferon Cytokine Res*. 2011;31:5-11.
3. Casey G, Neville PJ, Plummer SJ, et al. RNASEL Arg462Gln variant is implicated in up to 13% of prostate cancer cases. *Nat Genet*. 2002;32:581-583.
4. Chakrabarti A, Jha BK, Silverman RH. New insights into the role of RNase L in innate immunity. *J Interferon Cytokine Res*. 2011;31:49-57.
5. Rennert H, Bercovich D, Hubert A, et al. A novel founder mutation in the RNASEL gene, 471delAAAG, is associated with prostate cancer in Ashkenazi Jews. *Am J Hum Genet*. 2002;71:981-984.
6. Rokman A, Ikonen T, Seppala EH, et al. Germline alterations of the RNASEL gene, a candidate HPC1 gene at 1q25, in patients and families with prostate cancer. *Am J Hum Genet*. 2002;70:1299-1304.
7. Cairns P, Tokino K, Eby Y, Sidransky D. Homozygous deletions of 9p21 in primary human bladder tumors detected by comparative multiplex polymerase chain reaction. *Cancer Res*. 1994;54:1422-1424.
8. Slattery ML, Lundgreen A, Bondurant KL, Wolff RK. Interferon-signaling pathway: associations with colon and rectal cancer risk and subsequent survival. *Carcinogenesis*. 2011;32:1660-1667.
9. Diaz MO, Rubin CM, Harden A, et al. Deletions of interferon genes in acute lymphoblastic leukemia. *N Engl J Med*. 1990;322:77-82.
10. Green WB, Slovak ML, Chen IM, Pallavicini M, Hecht JL, Willman CL. Lack of IRF-1 expression in acute promyelocytic leukemia and in a subset of acute myeloid leukemias with del(5)(q31). *Leukemia*. 1999;13:1960-1971.
11. Hoek K, Rimm DL, Williams KR, et al. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res*. 2004;64:5270-5282.
12. Olopade OI, Buchhagen DL, Malik K, et al. Homozygous loss of the interferon genes defines the critical region on 9p that is deleted in lung cancers. *Cancer Res*. 1993;53:2410-2415.
13. Kulaeva OI, Draghici S, Tang L, Kraniak JM, Land SJ, Tainsky MA. Epigenetic silencing of multiple interferon pathway genes after cellular immortalization. *Oncogene*. 2003;22:4118-4127.
14. Reu FJ, Bae SI, Cherkassky L, et al. Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. *J Clin Oncol*. 2006;24:3771-3779.
15. Reu FJ, Leaman DW, Maitra RR, et al. Expression of RASSF1A, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. *Cancer Res*. 2006;66:2785-2793.
16. Dauer M, Schad K, Junkmann J, et al. IFN-alpha promotes definitive maturation of dendritic cells generated by short-term culture of monocytes with GM-CSF and IL-4. *J Leukoc Biol*. 2006;80:278-286.
17. Radvanyi LG, Banerjee A, Weir M, Messner H. Low levels of interferon-alpha induce CD86 (B7.2) expression and accelerates dendritic cell maturation from human peripheral blood mononuclear cells. *Scand J Immunol*. 1999;50:499-509.
18. Santini SM, Lapenta C, Logozzi M, et al. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J Exp Med*. 2000;191:1777-1788.
19. Tough DF. Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. *Leuk Lymphoma*. 2004;45:257-264.
20. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. 2006;6:836-848.
21. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.
22. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol*. 2011;29:235-271.
23. Borden EC, Sen GC, Uze G, et al. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov*. 2007;6:975-990.
24. Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci U S A*. 1994;91:1198-1205.
25. Bekisz J, Schmeisser H, Hernandez J, Goldman ND, Zoon KC. Human interferons alpha, beta and omega. *Growth Factors*. 2004;22:243-251.
26. Kotenko SV. IFN-lambdas. *Curr Opin Immunol*. 2011;23:583-590.
27. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*. 2004;202:8-32.
28. Pestka S. The human interferon-alpha species and hybrid proteins. *Semin Oncol*. 1997;24 S9-4S9-17.
29. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol*. 1993;11:571-611.
30. Nakaji M, Yano Y, Ninomiya T, et al. IFN-alpha prevents the growth of preneoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat. *Carcinogenesis*. 2004;25:389-397.
31. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity*. 2006;25:349-360.
32. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373-384.
33. Khoo JJ, Forster S, Mansell A. Toll-like receptors as interferon-regulated genes and their role in disease. *J Interferon Cytokine Res*. 2011;31:13-25.
34. Sen GC, Sarkar SN. Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev*. 2005;16:1-14.
35. Onoguchi K, Yoneyama M, Fujita T. Retinoic acid-inducible gene-I-like receptors. *J Interferon Cytokine Res*. 2011;31:27-31.
36. McWhirter SM, Barbalat R, Monroe KM, et al. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J Exp Med*. 2009;206:1899-1911.
37. Pandey AK, Yang Y, Jiang Z, et al. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. *PLoS Pathog*. 2009;5:e1000500.
38. Watanabe T, Asano N, Fichtner-Feigl S, et al. NOD1 contributes to mouse host defense against *Helicobacter pylori* via induction of type I IFN and activation of the ISGF3 signaling pathway. *J Clin Invest*. 2010;120:1645-1662.
39. Fidock MD, Souberbielle BE, Laxton C, et al. The innate immune response, clinical outcomes, and ex vivo HCV antiviral efficacy of a TLR7 agonist (PF-4878691). *Clin Pharmacol Ther*. 2011;89:821-829.
40. Pashenkov M, Goess G, Wagner C, et al. Phase II trial of a toll-like receptor 9-activating oligonucleotide in patients with metastatic melanoma. *J Clin Oncol*. 2006;24:5716-5724.
41. Puig M, Tosh KW, Schramm LM, et al. TLR9 and TLR7 agonists mediate distinct type I IFN responses in humans and nonhuman primates in vitro and in vivo. *J Leukoc Biol*. 2012;91:147-158.
42. Maniatis T, Falvo JV, Kim TH, et al. Structure and function of the interferon-beta enhanceosome. *Cold Spring Harb Symp Quant Biol*. 1998;63:609-620.
43. Panne D, Maniatis T, Harrison SC. An atomic model of the interferon-beta enhanceosome. *Cell*. 2007;129:1111-1123.
44. Tailor P, Tamura T, Ozato K. IRF family proteins and type I interferon induction in dendritic cells. *Cell Res*. 2006;16:134-140.
45. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol*. 2006;6:644-658.
46. Tamura T, Yanai H, Savitsky D, Taniguchi T. The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol*. 2008;26:535-584.
47. Taniguchi T. Aimez-vous Brahms? A story capriccioso from the discovery of a cytokine family and its regulators. *Nat Immunol*. 2009;10:447-449.
48. Bukowski R, Ernstoff MS, Gore ME, et al. Pegylated interferon alfa-2b treatment for patients with solid tumors: a phase I/II study. *J Clin Oncol*. 2002;20:3841-3849.
49. Glue P, Fang JW, Rouzier-Panis R, et al. Pegylated interferon-alpha2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group. *Clin Pharmacol Ther*. 2000;68:556-567.
50. Grace M, Youngster S, Gitlin G, et al. Structural and biologic characterization of pegylated recombinant IFN- alpha2b. *J Interferon Cytokine Res*. 2001;21:1103-1115.
51. Monkarsh SP, Ma Y, Aglione A, et al. Positional isomers of monopegylated interferon alpha-2a: isolation, characterization, and biological activity. *Anal Biochem*. 1997;247:434-440.

52. Zeuzem S, Welsch C, Herrmann E. Pharmacokinetics of peginterferons. *Semin Liver Dis*. 2003;23(suppl 1):23-28.
53. Novick D, Orchansky P, Revel M, Rubinstein M. The human interferon-gamma receptor. Purification, characterization, and preparation of antibodies. *J Biol Chem*. 1987;262:8483-8487.
54. Ruzicka FJ, Jach ME, Borden EC. Binding of recombinant-produced interferon beta ser to human lymphoblastoid cells. Evidence for two binding domains. *J Biol Chem*. 1987;262:16142-16149.
55. Thomas C, Moraga I, Levin D, et al. Structural linkage between ligand discrimination and receptor activation by type I interferons. *Cell*. 2011;146:621-632.
56. Leaman DW, Chawla-Sarkar M, Jacobs B, et al. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. *J Interferon Cytokine Res*. 2003;23:745-756.
57. Trinchieri G. Type I interferon: friend or foe? *J Exp Med*. 2010;207:2053-2063.
58. Abramovich C, Shulman LM, Ratovitski E, et al. Differential tyrosine phosphorylation of the IFNAR chain of the type I interferon receptor and of an associated surface protein in response to IFN-alpha and IFN-beta. *EMBO J*. 1994;13:5871-5877.
59. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem*. 1998;67:227-264.
60. Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. *Nat Rev Immunol*. 2002;2:410-416.
61. Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells*. 2001;19:378-387.
62. Matsuyama T, Kimura T, Kitagawa M, et al. Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. *Cell*. 1993;75:83-97.
63. Fenner JE, Starr R, Cornish AL, et al. Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. *Nat Immunol*. 2006;7:33-39.
64. Tomita S, Ishibashi K, Hashimoto K, et al. Suppression of SOCS3 increases susceptibility of renal cell carcinoma to interferon-alpha. *Cancer Sci*. 2011;102:57-63.
65. Brierley MM, Fish EN. Review: IFN-alpha/beta receptor interactions to biologic outcomes: understanding the circuitry. *J Interferon Cytokine Res*. 2002;22:835-845.
66. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol*. 2005;5:375-386.
67. Kramer OH, Knauer SK, Greiner G, et al. A phosphorylation-acetylation switch regulates STAT1 signaling. *Genes Dev*. 2009;23:223-235.
68. Du Z, Shen Y, Yang W, Mecklenbrauker I, Neel BG, Ivashkiv LB. Inhibition of IFN-alpha signaling by a PKC- and protein tyrosine phosphatase SHP-2-dependent pathway. *Proc Natl Acad Sci U S A*. 2005;102:10267-10272.
69. Wu TR, Hong YK, Wang XD, et al. SHP-2 is a dual-specificity phosphatase involved in Stat1 dephosphorylation at both tyrosine and serine residues in nuclei. *J Biol Chem*. 2002;277:47572-47580.
70. Yi T, Pathak MK, Lindner DJ, Ketterer ME, Farver C, Borden EC. Anticancer activity of sodium stibogluconate in synergy with IFNs. *J Immunol*. 2002;169:5978-59785.
71. Muller P, Kuttenkeuler D, Gesellchen V, Zeidler MP, Boutros M. Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature*. 2005;436:871-875.
72. Kundu S, Fan K, Cao M, et al. Tyrosine phosphatase inhibitor-3 sensitizes melanoma and colon cancer to biotherapeutics and chemotherapeutics. *Mol Cancer Ther*. 2010;9:2287-2296.
73. Kundu S, Fan K, Cao M, et al. Novel SHP-1 inhibitors tyrosine phosphatase inhibitor-1 and analogs with preclinical anti-tumor activities as tolerated oral agents. *J Immunol*. 2010;184:6529-6536.
74. Purdy AK, Campbell KS. SHP-2 expression negatively regulates NK cell function. *J Immunol*. 2009;183:7234-7243.
75. Win-Piazza H, Schneeberger VE, Chen L, et al. Enhanced anti-melanoma efficacy of interferon alfa-2b via inhibition of Shp2. *Cancer Lett*. 2012;320:81-85.
76. Gomez JA, Sun W, Gama V, et al. The C-terminus of interferon gamma receptor beta chain (IFNgammaR2) has antiapoptotic activity as a Bax inhibitor. *Cancer Biol Ther*. 2009;8:1771-1786.
77. Mennechet FJ, Uze G. Interferon-lambda-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells. *Blood*. 2006;107:4417-4423.
78. van Boxel-Dezaire AH, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity*. 2006;25:361-372.
79. Cheon H, Yang J, Stark GR. The functions of signal transducers and activators of transcriptions 1 and 3 as cytokine-inducible proteins. *J Interferon Cytokine Res*. 2011;31:33-40.
80. Kong LY, Gelbard A, Wei J, et al. Inhibition of p-STAT3 enhances IFN-alpha efficacy against metastatic melanoma in a murine model. *Clin Cancer Res*. 2010;16:2550-2561.
81. Morrow AN, Schmeisser H, Tsuno T, Zoon KC. A novel role for IFN-stimulated gene factor 3II in IFN-gamma signaling and induction of antiviral activity in human cells. *J Immunol*. 2011;186:1685-1693.
82. Velazquez L, Mogensen KE, Barbieri G, Fellous M, Uze G, Pellegrini S. Distinct domains of the protein tyrosine kinase tyk2 required for binding of interferon-alpha/beta and for signal transduction. *J Biol Chem*. 1995;270:3327-3334.
83. Rani MR, Ransohoff RM. Alternative and accessory pathways in the regulation of IFN-beta-mediated gene expression. *J Interferon Cytokine Res*. 2005;25:788-798.
84. Jaitin DA, Roisman LC, Jaks E, et al. Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. *Mol Cell Biol*. 2006;26:1888-1897.
85. Slutzki M, Jaitin DA, Yehezkel TB, Schreiber G. Variations in the unstructured C-terminal tail of interferons contribute to differential receptor binding and biological activity. *J Mol Biol*. 2006;360:1019-1030.
86. Kumar KG, Liu J, Li Y, et al. Raf inhibitor stabilizes receptor for the type I interferon but inhibits its anti-proliferative effects in human malignant melanoma cells. *Cancer Biol Ther*. 2007;6:1437-1441.
87. Brierley MM, Fish EN. Stats: multifaceted regulators of transcription. *J Interferon Cytokine Res*. 2005;25:733-744.
88. Tomic J, Lichty B, Spaner DE. Aberrant interferon-signaling is associated with aggressive chronic lymphocytic leukemia. *Blood*. 2011;117:2668-2680.
89. Testoni B, Vollenkle C, Guerrieri F, Gerbal-Chaloin S, Blandino G, Levrero M. Chromatin dynamics of gene activation and repression in response to interferon alpha (IFN(alpha)) reveal new roles for phosphorylated and unphosphorylated forms of the transcription factor STAT2. *J Biol Chem*. 2011;286:20217-20227.
90. Hartman SE, Bertone P, Nath AK, et al. Global changes in STAT target selection and transcription regulation upon interferon treatments. *Genes Dev*. 2005;19:2953-2968.
91. Kaur S, Sassano A, Joseph AM, et al. Dual regulatory roles of phosphatidylinositol 3-kinase in IFN signaling. *J Immunol*. 2008;181:7316-7323.
92. David M, Petricoin 3rd E, Benjamin C, Pine R, Weber MJ, Larner AC. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science*. 1995;269:1721-1723.
93. Lu M, Zhang W, Li Y, et al. Interferon-alpha targets JAK2V617F-positive hematopoietic progenitor cells and acts through the p38 MAPK pathway. *Exp Hematol*. 2010;38:472-480.
94. Joshi S, Kaur S, Redig AJ, et al. Type I interferon (IFN)-dependent activation of Mnk1 and its role in the generation of growth inhibitory responses. *Proc Natl Acad Sci U S A*. 2009;106:12097-12102.
95. Shmulevitz M, Pan LZ, Garant K, Pan D, Lee PW. Oncogenic Ras promotes reovirus spread by suppressing IFN-beta production through negative regulation of RIG-I signaling. *Cancer Res*. 2010;70:4912-4921.
96. Hasan UA, Caux C, Perrot I, et al. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. *Proc Natl Acad Sci U S A*. 2007;104:8047-8052.
97. Katze MG, He Y, Gale Jr M. Viruses and interferon: a fight for supremacy. *Nat Rev Immunol*. 2002;2:675-687.
98. O'Brien PM, Saveria Campo M. Evasion of host immunity directed by papillomavirus-encoded proteins. *Virus Res*. 2002;88:103-117.
99. Rincon-Orozco B, Halec G, Rosenberger S, et al. Epigenetic silencing of interferon-kappa in human papillomavirus type 16-positive cells. *Cancer Res*. 2009;69:8718-8725.
100. Schiavoni G, Sistigu A, Valentini M, et al. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Res*. 2011;71:768-778.

101. Borden EC, Sidky YA, Erturk E, Wierenga W, Bryan GT. Protection from carcinogen-induced murine bladder carcinoma by interferons and an oral interferon-inducing pyrimidinone, bropirimine. *Cancer Res*. 1990;50:1071-1074.
102. Luth S, Schrader J, Zander S, et al. Chronic inflammatory IFN-gamma signaling suppresses hepatocarcinogenesis in mice by sensitizing hepatocytes for apoptosis. *Cancer Res*. 2011;71:3763-3771.
103. Tu SP, Quante M, Bhagat G, et al. IFN-gamma inhibits gastric carcinogenesis by inducing epithelial cell autophagy and T-cell apoptosis. *Cancer Res*. 2011;71:4247-4259.
104. Qin XQ, Tao N, Dergay A, et al. Interferon-beta gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice. *Proc Natl Acad Sci U S A*. 1998;95:14411-14416.
105. Sanceau J, Poupon MF, Delattre O, Sastre-Garau X, Wietzerbin J. Strong inhibition of Ewing tumor xenograft growth by combination of human interferon-alpha or interferon-beta with ifosfamide. *Oncogene*. 2002;21:7700-7709.
106. Reid TR, Merigan TC, Basham TY. Resistance to interferon-alpha in a mouse B-cell lymphoma involves DNA methylation. *J Interferon Res*. 1992;12:131-137.
107. Friedman RM, Yeh A, Gutman P, Contente S, Kenyon K. Reversion by deletion of transforming oncogene following interferon-beta and retinoic acid treatment. *J Interferon Cytokine Res*. 1997;17:647-651.
108. Tsai YC, Pestka S, Wang LH, et al. Interferon-beta signaling contributes to Ras transformation. *PLoS One*. 2011;6:e24291.
109. Belardelli F, Gresser I, Maury C, Maunoury MT. Antitumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. II. Role of host mechanisms. *Int J Cancer*. 1982;30:821-825.
110. Reid TR, Race ER, Wolff BH, Friedman RM, Merigan TC, Basham TY. Enhanced in vivo therapeutic response to interferon in mice with an in vitro interferon-resistant B-cell lymphoma. *Cancer Res*. 1989;49:4163-4169.
111. Reid LM, Minato N, Gresser I, Holland J, Kadish A, Bloom BR. Influence of anti-mouse interferon serum on the growth and metastasis of tumor cells persistently infected with virus and of human prostatic tumors in athymic nude mice. *Proc Natl Acad Sci U S A*. 1981;78:1171-1175.
112. Lesinski GB, Anghelina M, Zimmerer J, et al. The antitumor effects of IFN-alpha are abrogated in a STAT1-deficient mouse. *J Clin Invest*. 2003;112:170-180.
113. Badgwell B, Lesinski GB, Magro C, Abood G, Skaf A, Carson 3rd W. The antitumor effects of interferon-alpha are maintained in mice challenged with a STAT1-deficient murine melanoma cell line. *J Surg Res*. 2004;116:129-136.
114. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 1998;95:7556-7561.
115. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410:1107-1111.
116. Dunn GP, Bruce AT, Sheehan KC, et al. A critical function for type I interferons in cancer immunoediting. *Nat Immunol*. 2005;6:722-729.
117. Swann JB, Hayakawa Y, Zerafa N, et al. Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. *J Immunol*. 2007;178:7540-7549.
118. Diamond MS, Kinder M, Matsushita H, et al. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J Exp Med*. 2011;208:1989-2003.
119. Fuertes MB, Kacha AK, Kline J, et al. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8α+ dendritic cells. *J Exp Med*. 2011;208:2005-2016.
120. Burchert A, Cai D, Hofbauer LC, et al. Interferon consensus sequence binding protein (ICSBP; IRF-8) antagonizes BCR/ABL and down-regulates bcl-2. *Blood*. 2004;103:3480-3489.
121. Yamamoto M, Kato T, Hotta C, et al. Shared and distinct functions of the transcription factors IRF4 and IRF8 in myeloid cell development. *PLoS One*. 2011;6:e25812.
122. Havelange V, Pekarsky Y, Nakamura T, et al. IRF4 mutations in chronic lymphocytic leukemia. *Blood*. 2011;118:2827-2829.
123. Han J, Qureshi AA, Nan H, et al. A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer Res*. 2011;71:1533-1539.
124. Salaverria I, Philipp C, Oschlies I, et al. Translocations activating IRF4 identify a subtype of germinal center-derived B-cell lymphoma affecting predominantly children and young adults. *Blood*. 2011;118:139-147.
125. Bi X, Hameed M, Mirani N, Pimenta EM, Anari J, Barnes BJ. Loss of interferon regulatory factor 5 (IRF5) expression in human ductal carcinoma correlates with disease stage and contributes to metastasis. *Breast Cancer Res*. 2011;13:R111.
126. Khodarev NN, Roizman B, Weichselbaum RR. Molecular pathways: interferon/stat1 pathway: role in the tumor resistance to genotoxic stress and aggressive growth. *Clin Cancer Res*. 2012;18:3015-3021.
127. Luszczek W, Cheriyath V, Mekhail TM, Borden EC. Combinations of DNA methyltransferase and histone deacetylase inhibitors induce DNA damage in small cell lung cancer cells: correlation of resistance with IFN-stimulated gene expression. *Mol Cancer Ther*. 2010;9:2309-2321.
128. Weichselbaum RR, Ishwaran H, Yoon T, et al. An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. *Proc Natl Acad Sci U S A*. 2008;105:18490-18495.
129. Cheriyath V, Glaser KB, Waring JF, Baz R, Hussein MA, Borden EC. G1P3, an IFN-induced survival factor, antagonizes TRAIL-induced apoptosis in human myeloma cells. *J Clin Invest*. 2007;117:3107-3117.
130. Bektas N, Noetzel E, Veeck J, et al. The ubiquitin-like molecule interferon-stimulated gene 15 (ISG15) is a potential prognostic marker in human breast cancer. *Breast Cancer Res*. 2008;10:R58.
131. Cheriyath V, Leaman DW, Borden EC. Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *J Interferon Cytokine Res*. 2011;31:173-181.
132. Borden EC, Williams BR. Interferon-stimulated genes and their protein products: what and how? *J Interferon Cytokine Res*. 2011;31:1-4.
133. Brown TD, Goodman PJ, Fleming T, et al. Phase II trial of recombinant DNA gamma-interferon in advanced colorectal cancer: a Southwest Oncology Group study. *J Immunother*. 1991;10:379-382.
134. Waddell SJ, Popper SJ, Rubins KH, et al. Dissecting interferon-induced transcriptional programs in human peripheral blood cells. *PLoS One*. 2010;5:e9753.
135. Chawla-Sarkar M, Lindner DJ, Liu YF, et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis*. 2003;8:237-249.
136. Chen Q, Gong B, Mahmoud-Ahmed AS, et al. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. *Blood*. 2001;98:2183-2192.
137. Clarke N, Jimenez-Lara AM, Voltz E, Gronemeyer H. Tumor suppressor IRF-1 mediates retinoid and interferon anticancer signaling to death ligand TRAIL. *EMBO J*. 2004;23:3051-3060.
138. Chen JJ, Knudsen S, Mazin W, Dahlgaard J, Zhang BA. 71-gene signature of TRAIL sensitivity in cancer cells. *Mol Cancer Ther*. 2012;11:34-44.
139. Choi C, Jeong E, Benveniste EN. Caspase-1 mediates Fas-induced apoptosis and is up-regulated by interferon-gamma in human astrocytoma cells. *J Neurooncol*. 2004;67:167-176.
140. Crowder C, Dahle O, Davis RE, Gabrielsen OS, Rudikoff S. PML mediates IFN-alpha-induced apoptosis in myeloma by regulating TRAIL induction. *Blood*. 2005;105:1280-1287.
141. Ding Y, Lee JF, Lu H, Lee MH, Yan DH. Interferon-inducible protein IFIXalpha1 functions as a negative regulator of HDM2. *Mol Cell. Biol*. 2006;26:1979-1996.
142. Leaman DW, Chawla-Sarkar M, Vyas K, et al. Identification of X-linked inhibitor of apoptosis-associated factor-1 as an interferon-stimulated gene that augments TRAIL Apo2L-induced apoptosis. *J Biol Chem*. 2002;277:28504-28511.
143. Lee MG, Huh JS, Chung SK, et al. Promoter CpG hypermethylation and downregulation of XAF1 expression in human urogenital malignancies: implication for attenuated p53 response to apoptotic stresses. *Oncogene*. 2006;25:5807-5822.
144. Pizzoferrato E, Liu Y, Gambotto A, et al. Ectopic expression of interferon regulatory factor-1 promotes human breast cancer cell death and results in reduced expression of survivin. *Cancer Res*. 2004;64:8381-8388.
145. Regad T, Chelbi-Alix MK. Role and fate of PML nuclear bodies in response to interferon and viral infections. *Oncogene*. 2001;20:7274-7286.

146. Wu WS, Xu ZX, Hittelman WN, Salomoni P, Pandolfi PP, Chang KS. Promyelocytic leukemia protein sensitizes tumor necrosis factor alpha-induced apoptosis by inhibiting the NF-kappaB survival pathway. *J Biol Chem*. 2003;278:12294-12304.
147. Kempkensteffen C, Fritzsche FR, Johannsen M, et al. Down-regulation of the pro-apoptotic XIAP associated factor-1 (XAF1) during progression of clear-cell renal cancer. *BMC Cancer*. 2009;9:276.
148. Cheriyath V, Kuhns MA, Jacobs BS, et al. G1P3, an interferon- and estrogen-induced survival protein contributes to hyperplasia, tamoxifen resistance and poor outcomes in breast cancer. *Oncogene*. 2012;31:2222-2236.
149. Tahara Jr E, Tahara H, Kanno M, et al. G1P3, an interferon inducible gene 6-16, is expressed in gastric cancers and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell. *Cancer Immunol Immunother*. 2005;54:729-740.
150. Selleri C, Sato T, Del Vecchio L, et al. Involvement of Fas-mediated apoptosis in the inhibitory effects of interferon-alpha in chronic myelogenous leukemia. *Blood*. 1997;89:957-964.
151. Buechner SA, Wernli M, Harr T, Hahn S, Itin P, Erb P. Regression of basal cell carcinoma by intralesional interferon-alpha treatment is mediated by CD95 (Apo-1/Fas)-CD95 ligand-induced suicide. *J Clin Invest*. 1997;100:2691-2696.
152. Thomas M, Finnegan CE, Rogers KM, et al. STAT1: a modulator of chemotherapy-induced apoptosis. *Cancer Res*. 2004;64:8357-8364.
153. Ugurel S, Seiter S, Rappl G, Stark A, Tilgen W, Reinhold U. Heterogenous susceptibility to CD95-induced apoptosis in melanoma cells correlates with bcl-2 and bcl-x expression and is sensitive to modulation by interferon-gamma. *Int J Cancer*. 1999;82:727-736.
154. Bani MR, Nicoletti MI, Alkharouf NW, et al. Gene expression correlating with response to paclitaxel in ovarian carcinoma xenografts. *Mol Cancer Ther*. 2004;3:111-121.
155. Castelli JC, Hassel BA, Wood KA, et al. A study of the interferon antiviral mechanism: apoptosis activation by the 2-5A system. *J Exp Med*. 1997;186:967-972.
156. Zhou A, Paranjape J, Brown TL, et al. Interferon action and apoptosis are defective in mice devoid of 2′,5′-oligoadenylate-dependent RNase L. *EMBO J*. 1997;16:6355-6363.
157. Malathi K, Paranjape JM, Ganapathi R, Silverman RH. HPC1/RNASEL mediates apoptosis of prostate cancer cells treated with 2′,5′-oligoadenylates, topoisomerase I inhibitors, and tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res*. 2004;64:9144-9151.
158. Xiang Y, Wang Z, Murakami J, et al. Effects of RNase L mutations associated with prostate cancer on apoptosis induced by 2′,5′-oligoadenylates. *Cancer Res*. 2003;63:6795-6801.
159. Maier C, Haeusler J, Herkommer K, et al. Mutation screening and association study of RNASEL as a prostate cancer susceptibility gene. *Br J Cancer*. 2005;92:1159-1164.
160. Wiklund F, Jonsson BA, Brookes AJ, et al. Genetic analysis of the RNASEL gene in hereditary, familial, and sporadic prostate cancer. *Clin Cancer Res*. 2004;10:7150-7156.
161. Dong B, Kim S, Hong S, et al. An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. *Proc Natl Acad Sci U S A*. 2007;104:1655-1660.
162. Bartsch DK, Fendrich V, Slater EP, et al. RNASEL germline variants are associated with pancreatic cancer. *Int J Cancer*. 2005;117:718-722.
163. Kruger S, Silber AS, Engel C, et al. Arg462Gln sequence variation in the prostate-cancer-susceptibility gene RNASEL and age of onset of hereditary non-polyposis colorectal cancer: a case-control study. *Lancet Oncol*. 2005;6:566-572.
164. Kristiansen H, Gad HH, Eskildsen-Larsen S, Despres P, Hartmann R. The oligoadenylate synthetase family: an ancient protein family with multiple antiviral activities. *J Interferon Cytokine Res*. 2011;31:41-47.
165. Zhou A, Hassel BA, Silverman RH. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. *Cell*. 1993;72:753-765.
166. Shou J, Soriano R, Hayward SW, Cunha GR, Williams PM, Gao WQ. Expression profiling of a human cell line model of prostatic cancer reveals a direct involvement of interferon signaling in prostate tumor progression. *Proc Natl Acad Sci U S A*. 2002;99:2830-2835.
167. Felger JC, Cole SW, Pace TW, et al. Molecular signatures of peripheral blood mononuclear cells during chronic interferon-alpha treatment: relationship with depression and fatigue. *Psychol Med*. 2012;42:1591-1603.
168. Barber GN. The dsRNA-dependent protein kinase, PKR and cell death. *Cell Death Differ*. 2005;12:563-570.
169. Der SD, Yang YL, Weissmann C, Williams BR. A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. *Proc Natl Acad Sci U S A*. 1997;94:3279-3283.
170. Pindel A, Sadler A. The role of protein kinase R in the interferon response. *J Interferon Cytokine Res*. 2011;31:59-70.
171. Williams BR. PKR; a sentinel kinase for cellular stress. *Oncogene*. 1999;18:6112-6120.
172. Dar AC, Dever TE, Sicheri F. Higher-order substrate recognition of eIF2alpha by the RNA-dependent protein kinase PKR. *Cell*. 2005;122:887-900.
173. Dey M, Cao C, Dar AC, et al. Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell*. 2005;122:901-913.
174. Patel RC, Sen GC. PACT, a protein activator of the interferon-induced protein kinase, PKR. *EMBO J*. 1998;17:4379-4390.
175. Yoon CH, Lee ES, Lim DS, Bae YS. PKR, a p53 target gene, plays a crucial role in the tumor-suppressor function of p53. *Proc Natl Acad Sci U S A*. 2009;106:7852-7857.
176. Scheuner D, Patel R, Wang F, et al. Double-stranded RNA-dependent protein kinase phosphorylation of the alpha-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. *J Biol Chem*. 2006;281:21458-21468.
177. Meurs EF, Galabru J, Barber GN, Katze MG, Hovanessian AG. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A*. 1993;90:232-236.
178. Sharma B, Altman JK, Goussetis DJ, Verma AK, Platanias LC. Protein kinase R as mediator of the effects of interferon (IFN) gamma and tumor necrosis factor (TNF) alpha on normal and dysplastic hematopoiesis. *J Biol Chem*. 2011;286:27506-27514.
179. Hii SI, Hardy L, Crough T, et al. Loss of PKR activity in chronic lymphocytic leukemia. *Int J Cancer*. 2004;109:329-335.
180. Thakur CS, Jha BK, Dong B, et al. Small-molecule activators of RNase L with broad-spectrum antiviral activity. *Proc Natl Acad Sci U S A*. 2007;104:9585-9590.
181. George CX, Gan Z, Liu Y, Samuel CE. Adenosine deaminases acting on RNA, RNA editing, and interferon action. *J Interferon Cytokine Res*. 2011;31:99-117.
182. Hartner JC, Walkley CR, Lu J, Orkin SH. ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling. *Nat Immunol*. 2009;10:109-115.
183. Toth AM, Li Z, Cattaneo R, Samuel CE. RNA-specific adenosine deaminase ADAR1 suppresses measles virus-induced apoptosis and activation of protein kinase PKR. *J Biol Chem*. 2009;284:29350-29356.
184. Fensterl V, Sen GC. The ISG56/IFIT1 gene family. *J Interferon Cytokine Res*. 2011;31:71-78.
185. Sarkar SN, Sen GC. Novel functions of proteins encoded by viral stress-inducible genes. *Pharmacol Ther*. 2004;103:245-259.
186. Terenzi F, Hui DJ, Merrick WC, Sen GC. Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56. *J Biol Chem*. 2006;281:34064-34071.
187. Stawowczyk M, Van Scoy S, Kumar KP, Reich NC. The interferon stimulated gene 54 promotes apoptosis. *J Biol Chem*. 2011;286:7257-7266.
188. Guo J, Sen GC. Characterization of the interaction between the interferon-induced protein P56 and the Int6 protein encoded by a locus of insertion of the mouse mammary tumor virus. *J Virol*. 2000;74:1892-1899.
189. Gariglio M, Mondini M, De Andrea M, Landolfo S. The multifaceted interferon-inducible p200 family proteins: from cell biology to human pathology. *J Interferon Cytokine Res*. 2011;31:159-172.
190. Asefa B, Klarmann KD, Copeland NG, Gilbert DJ, Jenkins NA, Keller JR. The interferon-inducible p200 family of proteins: a perspective on their roles in cell cycle regulation and differentiation. *Blood Cells Mol Dis*. 2004;32:155-167.
191. Cresswell KS, Clarke CJ, Jackson JT, Darcy PK, Trapani JA, Johnstone RW. Biochemical and growth regulatory activities of the HIN-200 family member and putative tumor suppressor protein, AIM2. *Biochem Biophys Res Commun*. 2005;326:417-424.

192. DeYoung KL, Ray ME, Su YA, et al. Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. *Oncogene*. 1997;15:453-457.
193. Fujiuchi N, Aglipay JA, Ohtsuka T, et al. Requirement of IFI16 for the maximal activation of p53 induced by ionizing radiation. *J Biol Chem*. 2004;279:20339-20344.
194. Xin H, Pereira-Smith OM, Choubey D. Role of IFI 16 in cellular senescence of human fibroblasts. *Oncogene*. 2004;23:6209-6217.
195. Mazibrada J, De Andrea M, Ritta M, et al. In vivo growth inhibition of head and neck squamous cell carcinoma by the Interferon-inducible gene IFI16. *Cancer Lett*. 2010;287:33-43.
196. Chen IF, Ou-Yang F, Hung JY, et al. AIM2 suppresses human breast cancer cell proliferation in vitro and mammary tumor growth in a mouse model. *Mol Cancer Ther*. 2006;5:1-7.
197. Patsos G, Germann A, Gebert J, Dihlmann S. Restoration of absent in melanoma 2 (AIM2) induces G2/M cell cycle arrest and promotes invasion of colorectal cancer cells. *Int J Cancer*. 2010;126:1838-1849.
198. Xin H, Curry J, Johnstone RW, Nickoloff BJ, Choubey D. Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence. *Oncogene*. 2003;22:4831-4840.
199. Inbal B, Bialik S, Sabanay I, Shani G, Kimchi A. DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J Cell Biol*. 2002;157:455-468.
200. Inbal B, Cohen O, Polak-Charcon S, et al. DAP kinase links the control of apoptosis to metastasis. *Nature*. 1997;390:180-184.
201. Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. *Blood*. 1999;93:4347-4353.
202. Gade P, Roy SK, Li H, Nallar SC, Kalvakolanu DV. Critical role for transcription factor C/EBP-beta in regulating the expression of death-associated protein kinase 1. *Mol Cell Biol*. 2008;28:2528-2548.
203. Lee YR, Yuan WC, Ho HC, Chen CH, Shih HM, Chen RH. The Cullin 3 substrate adaptor KLHL20 mediates DAPK ubiquitination to control interferon responses. *EMBO J*. 2010;29:1748-1761.
204. Asselin-Paturel C, Trinchieri G. Production of type I interferons: plasmacytoid dendritic cells and beyond. *J Exp Med*. 2005;202:461-465.
205. Ito T, Amakawa R, Kaisho T, et al. Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J Exp Med*. 2002;195:1507-1512.
206. Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol*. 2001;31:2154-2163.
207. Siegel JP. Effects of interferon-gamma on the activation of human T lymphocytes. *Cell Immunol*. 1988;111:461-472.
208. Edwards BS, Hawkins MJ, Borden EC. Comparative in vivo and in vitro activation of human natural killer cells by two recombinant alpha-interferons differing in antiviral activity. *Cancer Res*. 1984;44:3135-3139.
209. Chaperot L, Blum A, Manches O, et al. Virus or TLR agonists induce TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. *J Immunol*. 2006;176:248-255.
210. Sato K, Hida S, Takayanagi H, et al. Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol*. 2001;31:3138-3146.
211. Guo B, Chang EY, Cheng G. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. *J Clin Invest*. 2008;118:1680-1690.
212. Henry T, Kirimanjeswara GS, Ruby T, et al. Type I IFN signaling constrains IL-17A/F secretion by gammadelta T cells during bacterial infections. *J Immunol*. 2010;184:3755-3767.
213. Dikopoulos N, Bertoletti A, Kroger A, Hauser H, Schirmbeck R, Reimann J. Type I IFN negatively regulates CD8+ T cell responses through IL-10-producing CD4+ T regulatory 1 cells. *J Immunol*. 2005;174:99-109.
214. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, et al. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer Res*. 2011;71:5101-5110.
215. Mescher MF, Curtsinger JM, Agarwal P, et al. Signals required for programming effector and memory development by CD8+ T cells. *Immunol Rev*. 2006;211:81-92.
216. Pamer E, Cresswell P. Mechanisms of MHC class I–restricted antigen processing. *Annu Rev Immunol*. 1998;16:323-358.
217. Rock KL, York IA, Saric T, Goldberg AL. Protein degradation and the generation of MHC class I–presented peptides. *Adv Immunol*. 2002;80:1-70.
218. Liberati AM, Horisberger MA, Garofani P, et al. Interferon-alpha-induced biologic modifications in patients with chronic myelogenous leukemia. *J Interferon Res*. 1994;14:349-355.
219. Witt PL, Storer BE, Bryan GT, et al. Pharmacodynamics of biological response in vivo after single and multiple doses of interferon-beta. *J Immunother Emphasis Tumor Immunol*. 1993;13:191-200.
220. Lee J, Li L, Gretz N, Gebert J, Dihlmann S. Absent in Melanoma 2 (AIM2) is an important mediator of interferon-dependent and -independent HLA-DRA and HLA-DRB gene expression in colorectal cancers. *Oncogene*. 2012;31:1242-1253.
221. Cramer LA, Nelson SL, Klemsz MJ. Synergistic induction of the Tap-1 gene by IFN-gamma and lipopolysaccharide in macrophages is regulated by STAT1. *J Immunol*. 2000;165:3190-3197.
222. Johnsen A, France J, Sy MS, Harding CV. Down-regulation of the transporter for antigen presentation, proteasome subunits, and class I major histocompatibility complex in tumor cell lines. *Cancer Res*. 1998;58:3660-3667.
223. Seliger B, Atkins D, Bock M, et al. Characterization of human lymphocyte antigen class I antigen-processing machinery defects in renal cell carcinoma lesions with special emphasis on transporter-associated with antigen-processing down-regulation. *Clin Cancer Res*. 2003;9:1721-1727.
224. Zhang L, Pagano JS. Interferon regulatory factor 7 mediates activation of Tap-2 by Epstein-Barr virus latent membrane protein 1. *J Virol*. 2001;75:341-350.
225. Storm van's Gravesande K, Layne MD, Ye Q, et al. IFN regulatory factor-1 regulates IFN-gamma-dependent cathepsin S expression. *J Immunol*. 2002;168:4488-4494.
226. Simmons DP, Wearsch PA, Canaday DH, et al. Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing. *J Immunol*. 2012;188:3116-3126.
227. Guadagni F, Schlom J, Johnston WW, et al. Selective interferon-induced enhancement of tumor-associated antigens on a spectrum of freshly isolated human adenocarcinoma cells. *J Natl Cancer Inst*. 1989;81:502-512.
228. Kastenmuller K, Wille-Reece U, Lindsay RW, et al. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. *J Clin Invest*. 2011;121:1782-1796.
229. Hance KW, Rogers CJ, Zaharoff DA, Canter D, Schlom J, Greiner JW. The antitumor and immunoadjuvant effects of IFN-alpha in combination with recombinant poxvirus vaccines. *Clin Cancer Res*. 2009;15:2387-2396.
230. Stagg J, Loi S, Divisekera U, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A*. 2011;108:7142-7147.
231. Cole KE, Strick CA, Paradis TJ, et al. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med*. 1998;187:2009-2021.
232. Marfaing-Koka A, Devergne O, Gorgone G, et al. Regulation of the production of the RANTES chemokine by endothelial cells. Synergistic induction by IFN-gamma plus TNF-alpha and inhibition by IL-4 and IL-13. *J Immunol*. 1995;154:1870-1878.
233. Mikhak Z, Fleming CM, Medoff BD, et al. STAT1 in peripheral tissue differentially regulates homing of antigen-specific Th1 and Th2 cells. *J Immunol*. 2006;176:4959-4967.
234. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol*. 2011;89:207-215.
235. Zhou Q, Zhao J, Al-Zoghaibi F, et al. Transcriptional control of the human plasma membrane phospholipid scramblase 1 gene is mediated by interferon-alpha. *Blood*. 2000;95:2593-2599.
236. Frasch SC, Henson PM, Kailey JM, et al. Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. *J Biol Chem*. 2000;275:23065-23073.
237. Silverman RH, Halloum A, Zhou A, et al. Suppression of ovarian carcinoma cell growth in vivo by the interferon-inducible plasma membrane protein, phospholipid scramblase 1. *Cancer Res*. 2002;62:397-402.

238. D'Cunha J, Knight Jr E, Haas AL, Truitt RL, Borden EC. Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc Natl Acad Sci U S A*. 1996;93:211-215.
239. Bogunovic D, Byun M, Durfee LA, et al. Mycobacterial disease and impaired IFN-gamma immunity in humans with inherited ISG15 deficiency. *Science*. 2012;337:1684-1688.
240. Giannakopoulos NV, Luo JK, Papov V, et al. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. *Biochem Biophys Res Commun*. 2005;336:496-506.
241. Padovan E, Terracciano L, Certa U, et al. Interferon stimulated gene 15 constitutively produced by melanoma cells induces e-cadherin expression on human dendritic cells. *Cancer Res*. 2002;62:3453-3458.
242. Ritchie KJ, Hahn CS, Kim KI, et al. Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. *Nat Med*. 2004;10:1374-1378.
243. Yan M, Luo JK, Ritchie KJ, et al. Ubp43 regulates BCR-ABL leukemogenesis via the type 1 interferon receptor signaling. *Blood*. 2007;110:305-312.
244. Zhang D, Zhang DE. Interferon-stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res*. 2011;31:119-130.
245. Potu H, Sgorbissa A, Brancolini C. Identification of USP18 as an important regulator of the susceptibility to IFN-alpha and drug-induced apoptosis. *Cancer Res*. 2010;70:655-665.
246. Wood LM, Pan ZK, Seavey MM, Muthukumaran G, Paterson Y. The ubiquitin-like protein, ISG15, is a novel tumor-associated antigen for cancer immunotherapy. *Cancer Immunol Immunother*. 2012;61:689-700.
247. Skaug B, Chen ZJ. Emerging role of ISG15 in antiviral immunity. *Cell*. 2010;143:187-190.
248. Mushinski JF, Nguyen P, Stevens LM, et al. Inhibition of tumor cell motility by the interferon-inducible GTPase MxA. *J Biol Chem*. 2009;284:15206-15214.
249. Lai KC, Chang KW, Liu CJ, Kao SY, Lee TC. IFN-induced protein with tetratricopeptide repeats 2 inhibits migration activity and increases survival of oral squamous cell carcinoma. *Mol Cancer Res*. 2008;6:1431-1439.
250. Katsoulidis E, Mavrommatis E, Woodard J, et al. Role of interferon α (IFNα)-inducible Schlafen-5 in regulation of anchorage-independent growth and invasion of malignant melanoma cells. *J Biol Chem*. 2010;285:40333-40341.
251. Siegrist F, Ebeling M, Certa U. The small interferon-induced transmembrane genes and proteins. *J Interferon Cytokine Res*. 2011;31:183-197.
252. Behr S, Schriever F. Engaging CD19 or target of an antiproliferative antibody 1 on human B lymphocytes induces binding of B cells to the interfollicular stroma of human tonsils via integrin alpha 4/beta 1 and fibronectin. *J Exp Med*. 1995;182:1191-1199.
253. Evans SS, Collea RP, Leasure JA, Lee DB. IFN-alpha induces homotypic adhesion and Leu-13 expression in human B lymphoid cells. *J Immunol*. 1993;150:736-747.
254. Tedder TF, Zhou LJ, Engel P. The CD19/CD21 signal transduction complex of B lymphocytes. *Immunol Today*. 1994;15:437-442.
255. Evans SS, Lee DB, Han T, Tomasi TB, Evans RL. Monoclonal antibody to the interferon-inducible protein Leu-13 triggers aggregation and inhibits proliferation of leukemic B cells. *Blood*. 1990;76:2583-2593.
256. Martensen PM, Justesen J. Small ISGs coming forward. *J Interferon Cytokine Res*. 2004;24:1-19.
257. Hatano H, Kudo Y, Ogawa I, et al. IFN-induced transmembrane protein 1 promotes invasion at early stage of head and neck cancer progression. *Clin Cancer Res*. 2008;14:6097-6105.
258. Lee J, Goh SH, Song N, et al. Overexpression of IFITM1 has clinicopathologic effects on gastric cancer and is regulated by an epigenetic mechanism. *Am J Pathol*. 2012;181:43-52.
259. Brem R, Hildebrandt T, Jarsch M, Van Muijen GN, Weidle UH. Identification of metastasis-associated genes by transcriptional profiling of a metastasizing versus a non-metastasizing human melanoma cell line. *Anticancer Res*. 2001;21:1731-1740.
260. Dvorak HF, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. *J Natl Cancer Inst*. 1989;81:497-502.
261. Sidky YA, Borden EC. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. *Cancer Res*. 1987;47:5155-5161.
262. Dinney CP, Bielenberg DR, Perrotte P, et al. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon-alpha administration. *Cancer Res*. 1998;58:808-814.
263. McCarty MF, Bielenberg D, Donawho C, Bucana CD, Fidler IJ. Evidence for the causal role of endogenous interferon-alpha/beta in the regulation of angiogenesis, tumorigenicity, and metastasis of cutaneous neoplasms. *Clin Exp Metastasis*. 2002;19:609-615.
264. von Marschall Z, Scholz A, Cramer T, et al. Effects of interferon alpha on vascular endothelial growth factor gene transcription and tumor angiogenesis. *J Natl Cancer Inst*. 2003;95:437-448.
265. Hiroi M, Mori K, Sakaeda Y, Shimada J, Ohmori Y. STAT1 represses hypoxia-inducible factor-1-mediated transcription. *Biochem Biophys Res Commun*. 2009;387:806-810.
266. Ray PS, Jia J, Yao P, Majumder M, Hatzoglou M, Fox PL. A stress-responsive RNA switch regulates VEGFA expression. *Nature*. 2009;457:915-919.
267. Reznikov LL, Puren AJ, Fantuzzi G, et al. Spontaneous and inducible cytokine responses in healthy humans receiving a single dose of IFN-alpha2b: increased production of interleukin-1 receptor antagonist and suppression of IL-1-induced IL-8. *J Interferon Cytokine Res*. 1998;18:897-903.
268. Singh RK, Gutman M, Llansa N, Fidler IJ. Interferon-beta prevents the upregulation of interleukin-8 expression in human melanoma cells. *J Interferon Cytokine Res*. 1996;16:577-584.
269. Feldman ED, Weinreich DM, Carroll NM, et al. Interferon gamma-inducible protein 10 selectively inhibits proliferation and induces apoptosis in endothelial cells. *Ann Surg Oncol*. 2006;13:125-133.
270. Yang J, Richmond A. The angiostatic activity of interferon-inducible protein-10/CXCL10 in human melanoma depends on binding to CXCR3 but not to glycosaminoglycan. *Mol Ther*. 2004;9:846-855.
271. Vestal DJ, Jeyaratnam JA. The guanylate-binding proteins: emerging insights into the biochemical properties and functions of this family of large interferon-induced guanosine triphosphatase. *J Interferon Cytokine Res*. 2011;31:89-97.
272. Guenzi E, Topolt K, Lubeseder-Martellato C, et al. The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J*. 2003;22:3772-3782.
273. Guenzi E, Topolt K, Cornali E, et al. The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J*. 2001;20:5568-5577.
274. Li M, Mukasa A, Inda M, et al. Guanylate binding protein 1 is a novel effector of EGFR-driven invasion in glioblastoma. *J Exp Med*. 2011;208:2657-2673.
275. Raffaella R, Gioia D, De Andrea M, et al. The interferon-inducible IFI16 gene inhibits tube morphogenesis and proliferation of primary, but not HPV16 E6/E7-immortalized human endothelial cells. *Exp Cell Res*. 2004;293:331-345.
276. Taylor KL, Leaman DW, Grane R, Mechti N, Borden EC, Lindner DJ. Identification of interferon-beta-stimulated genes that inhibit angiogenesis in vitro. *J Interferon Cytokine Res*. 2008;28:733-740.
277. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest*. 2010;120:1151-1164.
278. Indraccolo S. Interferon-alpha as angiogenesis inhibitor: learning from tumor models. *Autoimmunity*. 2010;43:244-247.
279. Borden EC. Interferons. In: Holland JF, Kufe D, Pollock R, eds. *Cancer Medicine* 7th ed. Philadelphia, Pa: BC Decker; 2006:733-743.
280. Chang E, Boyd A, Nelson CC, et al. Successful treatment of infantile hemangiomas with interferon-alpha-2b. *J Pediatr Hematol Oncol*. 1997;19:237-244.
281. Ezekowitz RA, Mulliken JB, Folkman J. Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. *N Engl J Med*. 1992;326:1456-1463.
282. Long-term follow-up of the Italian trial of interferon-alpha versus conventional chemotherapy in chronic myeloid leukemia. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. *Blood*. 1998;92:1541-1548.
283. Bonifazi F, de Vivo A, Rosti G, et al. Chronic myeloid leukemia and interferon-alpha: a study of complete cytogenetic responders. *Blood*. 2001;98:3074-3081.
284. Kantarjian HM, O'Brien S, Smith TL, et al. Treatment of Philadelphia chromosome-positive early chronic phase chronic myelogenous leukemia with daily doses of interferon alpha and low-dose cytarabine. *J Clin Oncol*. 1999;17:284-292.

285. Kurzrock R, Talpaz M, Gutterman JU. Hairy cell leukaemia: review of treatment. *Br J Haematol*. 1991;79(suppl 1):17-20.
286. Talpaz M. Interferon-alfa-based treatment of chronic myeloid leukemia and implications of signal transduction inhibition. *Semin Hematol*. 2001;38:22-27.
287. Hehlmann R, Lauseker M, Jung-Munkwitz S, et al. Tolerability-adapted imatinib 800 mg/d versus 400 mg/d versus 400 mg/d plus interferon-alpha in newly diagnosed chronic myeloid leukemia. *J Clin Oncol*. 2011;29:1634-1642.
288. Kiladjian JJ, Mesa RA, Hoffman R. The renaissance of interferon therapy for the treatment of myeloid malignancies. *Blood*. 2011;117:4706-4715.
289. Kreutzman A, Rohon P, Faber E, et al. Chronic myeloid leukemia patients in prolonged remission following interferon-alpha monotherapy have distinct cytokine and oligoclonal lymphocyte profile. *PLoS One*. 2011;6:e23022.
290. Simonsson B, Gedde-Dahl T, Markevarn B, et al. Combination of pegylated IFN-alpha2b with imatinib increases molecular response rates in patients with low- or intermediate-risk chronic myeloid leukemia. *Blood*. 2011;118:3228-3235.
291. Samanta AK, Lin H, Sun T, Kantarjian H, Arlinghaus RB. Janus kinase 2: a critical target in chronic myelogenous leukemia. *Cancer Res*. 2006;66:6468-6472.
292. Quintas-Cardama A, Kantarjian H, Manshouri T, et al. Pegylated interferon alfa-2a yields high rates of hematologic and molecular response in patients with advanced essential thrombocythemia and polycythemia vera. *J Clin Oncol*. 2009;27:5418-5424.
293. Gresser I, Maury C, Belardelli F. Anti-tumor effects of interferon in mice injected with interferon-sensitive and interferon-resistant Friend leukemia cells. VI. Adjuvant therapy after surgery in the inhibition of liver and spleen metastases. *Int J Cancer*. 1987;39:789-792.
294. Muller CR, Smeland S, Bauer HC, Saeter G, Strander H. Interferon-alpha as the only adjuvant treatment in high-grade osteosarcoma: long term results of the Karolinska Hospital series. *Acta Oncol*. 2005;44:475-480.
295. Kirkwood JM, Manola J, Ibrahim J, Sondak V, Ernstoff MS, Rao U. A pooled analysis of Eastern Cooperative Oncology Group and intergroup trials of adjuvant high-dose interferon for melanoma. *Clin Cancer Res*. 2004;10:1670-1677.
296. Mocellin S, Pasquali S, Rossi CR, Nitti D. Interferon alpha adjuvant therapy in patients with high-risk melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst*. 2010;102:493-501.
297. Eggermont AM, Suciu S, Santinami M, et al. Adjuvant therapy with pegylated interferon alfa-2b versus observation alone in resected stage III melanoma: final results of EORTC 18991, a randomised phase III trial. *Lancet*. 2008;372:117-126.
298. Singal AK, Freeman Jr DH, Anand BS. Meta-analysis: interferon improves outcomes following ablation or resection of hepatocellular carcinoma. *Aliment Pharmacol Ther*. 2010;32:851-858.
299. Bukowski RM. Cytokine combinations: therapeutic use in patients with advanced renal cell carcinoma. *Semin Oncol*. 2000;27:204-212.
300. Flanigan RC, Mickisch G, Sylvester R, Tangen C, Van Poppel H, Crawford ED. Cytoreductive nephrectomy in patients with metastatic renal cancer: a combined analysis. *J Urol*. 2004;171:1071-1076.
301. Rini BI, Halabi S, Rosenberg JE, et al. Phase III trial of bevacizumab plus interferon alfa versus interferon alfa monotherapy in patients with metastatic renal cell carcinoma: final results of CALGB 90206. *J Clin Oncol*. 2010;28:2137-2143.
302. Motzer RJ, Hutson TE, Tomczak P, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol*. 2009;27:3584-3590.
303. Rohatiner AZ, Gregory WM, Peterson B, et al. Meta-analysis to evaluate the role of interferon in follicular lymphoma. *J Clin Oncol*. 2005;23:2215-2223.
304. Shah SU, Kaliki S, Kim HJ, Lally SE, Shields JA, Shields CL. Topical interferon alfa-2b for management of ocular surface squamous neoplasia in 23 cases: outcomes based on American Joint Committee on Cancer classification. *Arch Ophthalmol*. 2012;130:159-164.
305. Kirkwood JM, Bryant J, Schiller JH, Oken MM, Borden EC, Whiteside TL. Immunomodulatory function of interferon-gamma in patients with metastatic melanoma: results of a phase II-B trial in subjects with metastatic melanoma, ECOG study E 4987. Eastern Cooperative Oncology Group. *J Immunother*. 1997;20:146-157.
306. Kuebler JP, Goodman PJ, Brown TD, et al. Phase II study of continuous infusion recombinant gamma interferon in renal carcinoma. A Southwest Oncology Group study. *Invest New Drugs*. 1990;8:307-309.
307. Meyskens Jr FL, Kopecky KJ, Taylor CW, et al. Randomized trial of adjuvant human interferon gamma versus observation in high-risk cutaneous melanoma: a Southwest Oncology Group study. *J Natl Cancer Inst*. 1995;87:1710-1713.
308. Cho HI, Lee YR, Celis E. Interferon gamma limits the effectiveness of melanoma peptide vaccines. *Blood*. 2011;117:135-144.
309. Zaidi MR, Davis S, Noonan FP, et al. Interferon-gamma links ultraviolet radiation to melanomagenesis in mice. *Nature*. 2011;469:548-553.
310. Liu J, Hamrouni A, Wolowiec D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood*. 2007;110:296-304.
311. Taube JM, Anders RA, Young GD, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med*. 2012;4:127 ra37.
312. Link BK, Ballas ZK, Weisdorf D, et al. Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma. *J Immunother*. 2006;29:558-568.
313. Prins RM, Craft N, Bruhn KW, et al. The TLR-7 agonist, imiquimod, enhances dendritic cell survival and promotes tumor antigen-specific T cell priming: relation to central nervous system antitumor immunity. *J Immunol*. 2006;176:157-164.
314. Yan J, Chen SL, Wang HN, Wu TX. Meta-analysis of 5% imiquimod and 0.5% podophyllotoxin in the treatment of condylomata acuminata. *Dermatology*. 2006;213:218-223.
315. Gaspari A, Tyring SK, Rosen T. Beyond a decade of 5% imiquimod topical therapy. *J Drugs Dermatol*. 2009;8:467-474.
316. Friedberg JW, Kelly JL, Neuberg D, et al. Phase II study of a TLR-9 agonist (1018 ISS) with rituximab in patients with relapsed or refractory follicular lymphoma. *Br J Haematol*. 2009;146:282-291.
317. Triozzi PL, Aldrich W, Ponnazhagan S. Inhibition and promotion of tumor growth with adeno-associated virus carcinoembryonic antigen vaccine and Toll-like receptor agonists. *Cancer Gene Ther*. 2011;18:850-858.
318. Critchley-Thorne RJ, Yan N, Nacu S, Weber J, Holmes SP, Lee PP. Down-regulation of the interferon signaling pathway in T lymphocytes from patients with metastatic melanoma. *PLoS Med*. 2007;4:e176.
319. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer J*. 2010;16:399-403.

Tanja Meyer Tamguney, W. Michael Korn, and Frank McCormick

54

Gene Therapy and Oncolytic Viruses

Introduction

Genetic alterations are the driving force behind cancer development and progression. It follows that cancer could potentially be treated by correcting these alterations using gene therapy or by agents that kill cells by mechanisms based on these genetic alterations. Approaches of these kinds have a number of potential advantages. Vectors and viruses can be engineered in countless ways to achieve specificity and potency (Table 54-1) and can be designed and tested using tools that are routine in contemporary molecular biology laboratories. For these reasons, a dazzling array of creative concepts has been described over the past 20 years. However, few of these have been tested extensively in the clinic, and none has yet completed a successful Phase III clinical trial in the United States or Europe. Therefore, gene therapy and oncolytic viral therapy have not yet lived up to their promise and have not yet entered the mainstream of medical oncology, by any means. In this chapter, we discuss the potential and challenges of these novel technologies. We focus on the main strategies that have been evaluated clinically. These include the design of agents that kill cancer cells by gene replacement or by disruption of oncogenic signaling pathways. For example, therapeutic agents have been developed that reintroduce the wild-type p53 tumor suppressor gene or destroy RNA encoding oncogenic K-RAS. The delivery of toxic genes and genes that convert prodrugs into toxic metabolites has undergone extensive clinical testing and has produced exciting results. Furthermore, we highlight strategies that aim at modulating the immune response in order to achieve anticancer effects. Finally, we discuss the basic and clinical aspects of the use of replication-competent viruses, either in their natural configuration or genetically modified, to selectively kill cancer cells (Table 54-2).

Killing Cancer Cells by Gene Replacement and Gene Knockout

Gene Replacement

Cancers develop through loss of function of key regulatory genes known as tumor suppressors as well as through activation of proto-oncogenes. Loss of *p14*, *p16*, *p53*, or *PTEN* occurs at high frequencies in most tumor types. Loss of Adenomatous Polyposis Coli (*APC*) occurs in the majority of colorectal cancers, and loss of *RB* is frequent in small-cell lung cancers, among many other examples.[1,2] Mutations or deletions inactivate products of these genes, or their expression is suppressed by hypermethylation. Clonal evolution of tumors depends on loss of these functions: replacement of functional versions of these genes should therefore reverse this process. Indeed, when tumor suppressors are reexpressed in tumor cells by gene delivery, tumor cells die or growth is arrested. This has been shown for *APC*, *p16*, *p21*, *p27*, *RB*, *p14ARF*, *p53*, *PTEN*, *APC*, and *BRCA1*, *BRCA2*, among others.[3]

In contrast, normal cells receiving additional copies of these genes appear to be unaffected, at least in the cases that have been tested so far. This has been documented most clearly for p53: delivery of this tumor suppressor to normal bronchial epithelial cells had no effect on cell growth, suggesting a therapeutic window of more than 2 orders of magnitude.[4] This may be because tumor cells have additional defects that make them more sensitive to the effects of reexpressing p53. For example, almost all tumor cells have a defect in the RB checkpoint and therefore are less able to undergo growth arrest. Normal cells, in contrast, undergo G_1 arrest when p53 is active, and this can protect them from apoptosis. In addition, the negative regulator of p53,

Table 54-1 Gene Therapy and Oncolytic Viruses—Basic Concepts

		Class	Example
Vectors	Viral	Naturally cancer-selective viruses	Reovirus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles, vaccinia virus
		Engineered viruses	Adenovirus: ONYX015; herpes simplex virus (HSV): OncoVEXGM-CSF; vaccinia virus: JX-594
	Nonviral	Bacteria	*Listeria monocytogenes*: CRS-207
		Plasmids	Plasmid encoding IL-12
		RNAi	siRNA against RRM2: CALAA-01
Strategies to achieve tumor killing	Cell lysis/killing via viral replication		All replication-competent viruses
	Direct targeting of tumor genetics: knockdown of oncogenes or essential pathway components, reexpression of tumor suppressor genes		KRasG12D: siG12D LODER; p53: AdCMV-p53
	Delivery of cytotoxic or pro-apoptotic genes		Bcl-2; Survivin
	Delivery of immune-stimulatory genes		GM-CSF: JX594, OncoVEXGM-CSF; TNF: TNFerade
	Activation of innate and adaptive immunity, induction of memory		OncoVEXGM-CSF, JX594
	Delivery of anti-anti angiogenic genes and enzymes		Anti-FLT1 ribozyme
	Delivery of prodrug converting enzymes		Thymidine kinase, carboxylesterase, cytosinedeaminase
	Delivery of transporters		Sodium-iodide symporter (NIS)
	Chemosensitization by viral replication		All replication-competent viruses
Strategies to achieve tumor selectivity	Vector size: preferential exit from leaky tumor vasculature (fenestrations of ~100-400 nm); enhanced permeability retention effect (EPR)		CALAA-01 (70-nm diameter); vaccinia virus (200 nm)
	Vector engineering to match specific tumor genetics		Rb: adenovirus Delta24
	Targeting cell surface receptors overexpressed by cancer cells for delivery		Transferrin receptor: CALAA-01 siRNA delivering nanoparticles with transferrin as a targeting ligand
			$\alpha_v\beta$-Integrin: Adenovirus RGD
	Deletion of viral genes inducing S-phase, and/or other genes required for replication in quiescent cells		Adenovirus: E1A and E1B deletions; vaccinia: thymidine kinase deletion
	Use of tumor- and/or tissue-specific promoters		Adenovirus CG0070: E2F-1 driven expression of E1A

FLT1, Fms-like tyrosine kinase 1, VEGFR1; *RGD*, Arg-Gly-Asp; *RRM2*, ribonucleotide reductase subunit M2.

Mdm2, is itself a p53 effector.[5] In tumor cells lacking p53, mdm2 levels are often low. In normal cells, Mdm2 constantly degrades p53 and maintains low p53 activity. Therefore, p53 expressed ectopically in normal cells is degraded, whereas in cancer cells it is relatively stable. In addition, p53 promotes a bystander effect on uninfected tumor cells, possibly through antiangiogenesis,[6] secretion of soluble proapoptotic proteins,[7] and immune upregulation.[8,9] In agreement with these theoretical considerations, clinical safety and some evidence for anti-tumor activity of an adenoviral vector expresses wild-type p53 under the control of a Rous-sarcoma-virus promoter (Gendicine) have been demonstrated and led to approval of this agent in China for use in head and neck cancer.[10] Fever and flu-like symptoms were the main adverse events; no severe side effects occurred.[10] Long-lasting responses with this agent were observed in studies in patients with head and neck cancer. Very high response rates, including 64% complete responses, were observed following intratumoral injection of Gendicine in combination with radiation therapy.[11,12] However, a similar virus that delivers wild-type p53 under the control of a constitutively active CMV promoter (AdCMV-p53; Advexin) failed to complete a Phase III clinical trial successfully in the United States, and development of this agent stopped in 2008. This example illustrates the fact that poor delivery systems and inefficient access to tumor cells have limited the potential of gene-replacement therapy for treating cancer, despite compelling theoretical arguments for its development.

Gene Knockout

Antisense

Activation of proto-oncogenes such as *Ras*, *B-Raf*, *Myc*, or *EGFR* through various mechanisms is a key factor in carcinogenesis. For example, mutations of the *Ras* oncogene occur in many types, including pancreatic cancer, where about 90% of cases carry a mutation of the K-ras gene.[13,14] Knockout of oncogenes driving tumor development in transgenic mouse models of cancer can lead to complete regression, confirming

Table 54-2 Cancer-Selective Viruses in Clinical Trials

Virus Genus	Virus Product Name/Strain	Genome	Basis of Selectivity	(Other) Genetic Modification(s)	Clinical Phase	Route of Administration	Ref.
Enterovirus: Coxsackievirus A21, CVA21	CAVATAK	ssRNA$^+$	High levels of CAV21 receptors (ICAM1, DAF) expressed by cancer cells	None	Phase II malignant melanoma	Intratumoral	NCT00832559
Newcastle Disease Virus (NDV)	NDV-HUJ	ssRNA$^-$	Defective IFN response in transformed cells	None	Phase I/II glioblastoma, sarcoma, neuroblastoma	IV	122 NCT01174537
Parvovirus H-1 (apathogenic rat virus)	ParvOryx01	ssDNA	Defective IFN response in transformed cells; viral replication only in proliferating cells	None	Phase I/IIa glioblastoma multiforme	Intratumoral/ intracerebral or IV/intracerebral	123,124 NCT01301430
Reovirus		dsRNA	PKR suppression by Ras and oncogenic EGFR	None			
	Respiratory enteric orphan virus (REOLYSIN)				Phase II NSCLC; Phase II prostate cancer; Phase II colorectal cancer; Phase II pancreatic adenocarcinoma; Phase II melanoma; Phase II lung squamous cell carcinoma; Phase II head and neck carcinoma; Phase III head and neck carcinoma	IV	50,125 126 NCT00861627; NCT01619813; NCT01622543; NCT00998322; NCT00984464; NCT00998192; NCT00753038; NCT01166542
	Wild-type reovirus				Phase II fallopian tube cancer, ovarian cancer Primary peritoneal cavity cancer; Phase II pancreatic cancer	IV	NCT01199263; NCT01280058
Seneca-valley virus/ senecavirus	Senecavirus-001, NTX-010	ssRNA$^+$			Phase II NSCLC	IV	NCT01017601
Adenovirus	CG0070	DNA	E2F-1 promoter-driven expression of E1A	GM-CSF$^+$	Phase II/III Non–muscle-invasive bladder cancer	Bladder instillation	127 NCT01438112
Herpes Simplex Virus	OncoVEX GM-CSF	DNA	ICP34.5$^-$ (ICP34.5 related to eIF2 phosphatase regulatory subunit GADD34, involved in translational regulation)	ICP47$^-$ (immune stimulating, as ICP47 blocks antigen processing in HSV-infected cells), GM-CSF$^+$ (immune stimulating)	Phase III Malignant melanoma	Intratumoral	128,129 NCT01368276, NCT00769704

Continued

Table 54-2 Cancer-Selective Viruses in Clinical Trials—cont'd

Virus Genus	Virus Product Name/Strain	Genome	Basis of Selectivity	(Other) Genetic Modification(s)	Clinical Phase	Route of Administration	Ref.
Vaccinia		DNA	Natural tumor selectivity: permeable tumor vasculature; selective replication in metabolically active tumor environment Replication activated by epidermal growth factor receptor EGFR/Ras pathway signaling; cancer cell resistance to IFNs				65,130
	GL-ONC1, (attenuated Lister strain)		Thymidine kinase$^-$	Luciferase-GFP fusion$^+$ LacZ$^+$ GusA$^+$ Hemagglutinin$^-$	Phase I/II Peritoneal carcinomatosis	Intraperitoneal	131 NCT01443260
	JX-594, (Wyeth strain)		Thymidine kinase$^-$	GM-CSF, B-galactosidase	Ph2 Hepatocellular carcinoma; Ph1/2 colorectal carcinoma	IV	67,68,132 NCT00554372, NCT01171651, NCT01387555, NCT01394939

Phase I/II, Phase II, or Phase III clinical trials. June 2012. Clinicaltrials.gov.
EGFR, epidermal growth factor receptor; *GFP,* green fluorescent protein; *GusA,* β-glucuronidase; *ICAM1,* intercellular adhesion molecule 1; *IFN,* interferon; *IV,* intravenous; *DAF,* decay accelerating factor; *GM-CSF,* granulocyte macrophage colony-stimulating factor; *LacZ,* β-galactosidase; *NSCLC,* non–small-cell lung cancer; *PKR,* protein kinase R.

their potential value as drug targets.[15] Antisense technology has been studied as a strategy to knock down expression of oncogenes. This approach is based on the possibility of inhibiting transcription of a particular mRNA by transfecting short double-stranded DNA oligonucleotides into target cells that bind and inactivate the target mRNA in a sequence-specific manner.[16] The development of oligonucleotides with modified DNA backbone that increases the stability of the molecule in vivo allowed for the development of clinical protocols involving intravenous application of antisense molecules targeting, for example, mutant Kras or the anti-apoptotic genes *BCL-2* and *Survivin*.[17] Clinical trials with oblimersen sodium (G3139), an antisense oligonucleotide directed against BCL-2, have demonstrated increased survival of patients with advanced melanoma in combination with chemotherapy compared to chemotherapy alone.[18] An interesting variant of the antisense approach that is currently being tested clinically is GRN163L, a lipid-modified 13-mer DNA-oligonucleotide that acts as a telomerase RNA template antagonist.[19]

RNAi

The discovery of the possibility of silencing gene expression in mammalian cells with high efficiency using RNA interference (RNAi) has spurred renewed interest in the gene knock-down as a therapeutic strategy. This approach, first described in the worm *Caenorhabditis elegans*, takes advantage of a cellular gene silencing machinery that involves the RNA-induced silencing complex (RISC) and degrades double-stranded RNAs with high efficacy.[20] The introduction of short RNAs with complementary sequence to any cellular transcript activates this mechanism. RNAi can silence target genes with high efficacy. However, off-target effects that result in sequence-specific though unpredictable knock-down of additional genes represent a potential problem that has not been fully resolved at this point.[21] Nevertheless, novel targeting and delivery mechanisms have been developed that hold the promise of therapeutic application of RNAi-based agents in cancer.[20] For instance, a recently reported nanoparticle-based delivery system shows great promise for systemic delivery of siRNA. These synthetic nanoparticles consist of a cyclodextrin-based polymer, a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells, a hydrophilic polymer (polyethylene glycol [PEG] used to promote nanoparticle stability in biological fluids), and siRNA designed to reduce the expression of the Ribonucleotide Reductase subunit M2 (RRM2). Tumor-specific targeting is achieved via the interaction of the TF targeting ligand with TFR, which is known to be upregulated in malignant cells.[22] Moreover, the size of these nanoparticles, about 70 nm in diameter, favors their exit from the bloodstream in leaky tumor vasculature and accumulation in the tumor bed via the enhanced permeability and retention (EPR) effect. RRM2, an enzyme catalyzing a rate-limiting step in DNA synthesis, is an established anticancer target.[23] These particles (CALAA-01), currently in Phase I clinical studies, have been administered systemically to humans and have shown specific gene inhibition—a reduction in both mRNA and protein.[24]

Another well-established anticancer target is the *KRAS* oncogene. Most pancreatic adenocarcinomas are caused by a somatic mutation in *KRAS*, most commonly *KRAS G12D*. In vitro and animal studies suggest that selective inhibition of the mutant, but not the WT *KRAS* expression, leads to apoptosis of targeted cancer cells. An siRNA specifically targeting mRNA encoding KRAS G12D, coated by a biodegradable polymeric matrix, siG12D LODER (Local Drug EluteR), is currently in Phase 0/I to evaluate its safety and tolerability. Apart from these two particular RNAi therapeutics, at least another six are currently undergoing clinical testing, thanks to recent advancements in the understanding of RNAi biology and in the areas of RNAi specificity, stability, and delivery. Most are delivered employing synthetic carriers, such as cationic liposomes, anionic liposomes, and polymeric particles. As more attention is focused on safe and effective methods for delivering siRNA to tumors, the clinical value of this approach will likely increase dramatically. siRNA-based therapy has the potential of addressing undruggable oncogene targets, combinatorial approaches to cancer cell killing, and drug resistance, but this potential will not be realized until the challenges of delivery and uptake have been fully addressed.

Killing Cancer Cells by Delivering Prodrug Converting Enzymes and Other Enzymes

For more than 60 years chemotherapeutic agents have been used for the treatment of cancer. However, their use is often limited by damage to normal cells, drug resistance, and low chemical stability. One strategy to overcome limitations of classical chemotherapeutic agents is the use of prodrugs. A prodrug is a fairly nontoxic compound that needs to be transformed before acting as a pharmacon. Such a transformation can be catalyzed by endogenous enzymes, in which case tissue distribution of such endogenous enzymes dictates where the active pharmacon is produced. Alternatively, gene-directed enzyme prodrug therapy (GDEPT) can specifically deliver such enzymes to diseased cells where they can activate nontoxic prodrugs into toxic agents. Progress in this field has been reviewed recently by Duarte and colleagues.[25]

Thymidine Kinase

The first GDEPT system described was the thymidine kinase gene of the herpes simplex virus (HSV-TK) in combination with the prodrug nucleoside analog ganciclovir. The enzyme thymidine kinase (TK) is naturally present in bacteria, viruses, and mammals, where it is involved in the salvage pathway of nucleotide biosynthesis. Thus, high TK activity is found in proliferating cells such as cancer cells. TK also converts ganciclovir to ganciclovir monophosphate, which is subsequently converted by cellular kinases into the toxic ganciclovir triphosphate nucleotide. The HSV-TK is three orders of magnitude more efficient than any human kinase catalyzing this first activation step. Hence, several gene therapy approaches combining HSV-TK and ganciclovir have been developed.

For instance, retroviral vectors were used to deliver HSV-TK in a brain tumor model. Because retroviruses integrate only in proliferating cells, gene delivery and expression would be tumor selective in the context of normal, nonproliferating brain cells.[26] A further advantage of this approach was thought to result from the "bystander effect," the killing of uninfected neighboring cells that occurs when HSV-TK–expressing cells are exposed to ganciclovir, which can be observed in vitro and in vivo.[27] To increase transduction of target cells with retroviruses, virus-producing cells (VPCs) were used to inoculate target tumors instead of virus suspension. Preclinical studies in a rat glioma model demonstrated that this delivery of a retrovirus expressing HSV-TK resulted in high transduction levels and frequent tumor regressions following ganciclovir administration.[28] Initial clinical studies were promising and demonstrated in responses of small glioblastomas following VPC injection.[29] However, when standard therapy (surgical resection and radiotherapy) was compared to standard therapy plus injection of retrovirus-producing cells in a Phase III trial, no differences in progression-free and overall survival were observed between the two groups.[30] Smaller, preoperative studies suggested that lack of transduction of tumor cells is the dominant reason for the failure of this approach.[31] Despite the disappointing results of this trial, it represents an early and innovative effort to use gene therapy to kill cancer cells selectively.

Cytosine Deaminase

In contrast to thymidine kinase, the enzyme cytosine deaminase (CD) is not present in mammalian cells, but in several bacteria and fungi. It catalyzes the amidine hydrolysis of cytosine to uracil and ammonia, and several cytosine analogs such as halogenated cytosines are substrates as well. One such substrate is the prodrug 5′-fluorocytosine (5′-FC) which is activated by CD to 5′-fluorouracil (5′-FU). 5′-FU kills cells mainly by inhibition of thymidilate synthetase and by incorporation into DNA. The safety and efficacy of a CD and 5′-FC prodrug therapy are currently being assessed in Phase I/II trials in which 5′-FC and a recombinant *Bifidobacterium longum* (a live bacterium normally found in the digestive tract) that has been modified to produce CD are given orally to patients with various solid tumors.

Carboxylesterase

Another well-studied prodrug-converting enzyme is carboxylesterase (CE). CE expression is widely distributed in human tissues; nonetheless, targeted gene delivery to enhance CE expression specifically in tumor cells is a promising approach to increase the local availability of cytostatic drugs. CE is involved in the activating metabolism of various commonly used chemotherapeutics: It activates paclitaxel-2-ethylcarbonate to the microtubule-stabilizing agent paclitaxel/taxol and is involved in the multistep conversion of capecitabine to the pyrimidine analog 5′-fluorocytidine (5′-FC). Moreover, CE converts the relatively nontoxic camptothecin analog CPT-11/irinotecan to SN38, a potent topoisomerase I inhibitor. Because the human CE is comparatively inefficient in catalyzing this conversion, several gene therapeutic strategies using the more effective rabbit CE were developed and shown to be successful in tissue culture and animal models. Also, secreted mutants of this enzyme are being studied to enhance the spread of the toxic metabolite and thereby the bystander effect. For instance, Oosterhoff and colleagues show increased killing of colon carcinoma cells on infection with adenovirus expressing secreted CE (Ad5-Δ24-sCE) combined with CPT-11 treatment.[32] Even though CPT-11 also reduced viral replication, the overall cytotoxicity of virus combined with CPT-11 was still higher than cell killing achieved by the virus alone. Nonetheless, this exemplifies the complications of using chemotherapeutic agents in combination with oncolytic viruses; it is crucial to know the kinetics of both viral replication and drug metabolism to design a suitably timed treatment regimen. This is not only essential for oncolytic viruses as vectors for GDEPT, but for the combination of any oncolytic virus with chemotherapeutic agents.[33]

Sodium-Iodide Symporter

Not only prodrug-converting enzymes are being pursued as gene therapeutics. Several vectors have been developed

to deliver genes for various other enzymes, including the one for the sodium iodide symporter (*NIS*). The endogenously expressed NIS is an integral plasma membrane glycoprotein that mediates active I^- transport into thyroid follicular cells. The rationale for its use in gene therapy approaches is twofold: targeted expression of *NIS* in cancer cells renders these cells susceptible to uptake of radioactive iodine, which, depending on the isotope used, can be harnessed to kill and/or to image targeted cells. For instance, measles virus (MV) strains that have been engineered to express the human NIS (MV-NIS) have shown significant antitumor activity against various cancer lines and orthotopic xenografts. Expression of NIS in infected glioma cells resulted in effective concentration of radioactive iodine, which allowed for in vivo monitoring of localization of MV-NIS infection by measuring uptake of ^{123}I and led to a significant increase of MV-NIS antitumor activity.[34] A Phase I study is ongoing to establish the safety of intrapleural administration of this MV-NIS in patients with malignant pleural mesothelioma.

Another interesting approach is the retroviral delivery of a multidrug resistance pump (*MDR1*) gene into peripheral blood progenitor cells (PBPCs). The rationale behind this is not to kill cancer cells, but to prevent some of the toxicities of an intensive chemotherapy regimen in patients. PBPCs are isolated from the patient, genetically modified ex vivo, and transferred back into the patient. The hope is that introduction of the *MDR1* gene into the patients' PBPCs renders these cells and their offspring resistant to the toxic effects of certain chemotherapeutic agents, by pumping out chemotherapeutic agents before they can exert a cytotoxic effect.[35-39]

Many other strategies have been devised to express prodrug-converting enzymes, or other potentially toxic genes, in cancer cells selectively. Most of these depend on tumor-selective gene expression to drive the gene of interest, such as the promoters for the androgen receptor or prostate-specific antigen (PSA),[40-42] for tyrosinase (selective for melanoma),[43] or for alpha-lactalbumin (selective for breast cancer).[44] More generic tumor-selective–specific promoters include E2F-1, which is upregulated through loss of the RB checkpoint, and telomerase reverse transcriptase (TERT), which is also upregulated in most cancers.[45,46]

Genes That Boost the Immune System

Although oncolytic viruses can kill cancer cells directly via viral replication and by delivery of cytotoxic genes or prodrug-converting enzymes, it is becoming more and more evident that the great potential of viruses as gene therapeutic vectors lies in their immune-modulatory capability. This feature can be further enhanced by the addition of immune-modulatory genes into the viral genome. For virus-based therapies to be successful, both host immune system avoidance and potent immune stimulation need to be combined. One approach to boosting antitumor immune responses involves the administration of cytokines to increase the activity of immune effector cells systemically or to enhance the presentation of antigens in tumor cells themselves. A wide range of cytokine genes have been engineered into an equally wide range of vectors and viruses. IL-2, IL-4, and IL-12 have been studied extensively, as well as interferons, members of the tumor necrosis factor (TNF) family, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

TNF

TNFerade is an exciting example of this approach. This replication-incompetent adenovirus expresses TNF under the control of the Early Growth Response Factor 1 (EGRr-1) promoter, which is strongly activated in response to cellular stress such as iodizing radiation or chemotherapy.[47] TNFerade was injected in Phase I studies to up to 4×10^{11} pu (particle units) without dose-limiting toxicities occurring. In agreement with this favorable safety profile, serum TNF levels remained consistently low. However, in esophageal cancer, relatively high rates of thromboembolic complications occurred that were potentially induced by the study medication. This problem was not observed in trials of this agent in pancreatic cancer. In this disease the maximum tolerated dose (MTD) of TNFerade in combination with chemoradiation treatment was determined to be 4×10^{11} pu. At that dose level, clinical responses and evidence for prolonged median survival were seen in an interim analysis of a Phase III study in locally advanced pancreatic cancer in combination with chemoradiotherapy.[48]

GM-CSF

Of cytokines tested in oncolytic virus and vaccine models so far, GM-CSF seems to be the most potent at generating antitumor immune responses. HSV-, adenovirus-, and vaccinia virus–based vectors encoding GM-CSF have shown great promise in early clinical trials, and studies are still ongoing (see Table 54-2). Arming viruses with GM-CSF aims to activate the immune system primarily by attracting and activating dendritic cells (DCs). GM-CSF has been clinically tested in peptide and whole-cell vaccine strategies as well as oncolytic virus approaches. Notably, only virus-based

GM-CSF expression seems to generate a potent and sustained antitumor immune response, suggesting that the context in which GM-CSF is expressed is crucial. Virus-based GM-CSF expression and DC recruitment are tightly coupled to tumor cell death and subsequent release of a vast array of tumor antigens. Remarkably, for both HSV and vaccinia expressing GM-CSF, not only injected but also noninjected tumors responded. The studies mentioned earlier are among the first to combine oncolysis with immune system activation using armed, replication-competent viruses. Further exploitation of the immune system is a focus of current gene therapy and vaccine studies.[49]

Naturally Occurring Viruses That Replicate Selectively in Cancer Cells

Cancer cells provide an environment that is permissive for replication of a number of naturally occurring viruses. This is because checkpoints and defense mechanisms are disabled in cancer cells, allowing them to grow and survive and to evade detection by the immune system. In some cases these mechanisms are also used to defend normal cells against virus replication. Cancer cells may therefore be vulnerable to virus infection while normal cells are protected. Because infection usually leads to cell death, this vulnerability could potentially be exploited for cancer therapy. Indeed, several naturally occurring viruses are under clinical evaluation in a variety of cancer indications (see Table 54-2).

Reovirus

Reoviruses replicate selectively in many cancer cells. During infection of normal cells, their double-stranded RNA genomes activate a cellular protein kinase (PKR) that restricts viral replication by blocking translation of viral mRNA. For reasons that are unclear, this kinase activity is suppressed in cancer cells in which the Ras pathway is hyperactivated, allowing productive viral replication.[50] Based on this selectivity, and the capacity of reoviruses to replicate quickly and kill infected cells, reoviruses are undergoing clinical evaluation. One of these, Reolysin, is being tested in Canada, the United Kingdom, and the United States using clinical protocols that include local or systemic delivery of Reolysin as a monotherapy, and local delivery in combination with radiation therapy for patients with advanced cancers. Intravenous administration of this virus was well tolerated in a Phase I study. Although no objective tumor responses were observed, disease stabilization of up to 6 months was observed in a subset of patients.[51]

Vesicular Stomatitis Virus

The rhabdovirus vesicular stomatitis virus (VSV) has a single-stranded RNA genome. Selectivity for cancer cells is thought to be the result of their failure to elicit a protective interferon response, thus allowing lytic replication. VSV variants with mutations in the matrix (M) protein enhance VSV's effectiveness, at least in animal models.[52] Systemic delivery of VSV has been shown to be effective and safe against laboratory models of multifocal and invasive malignant gliomas.[53] M protein mutant viruses have also shown efficacy against prostate cancer cell lines and others.[52]

Measles

Measles viruses, like VSV, contain negative-stranded RNA genomes but are members of the paramyxovirus family. Replication-competent attenuated Edmonston B measles vaccine strain (MV-Edm) is nonpathogenic and has potent antitumor activity against several human tumors. The virus is selectively oncolytic, eliciting extensive cell-to-cell fusion and ultimately leading to cell death. An attenuated strain of MV has been genetically engineered to produce carcinoembryonic antigen, which can be used as a serum marker of virus replication.[12] This virus had potent antitumor activity against gliomas in vitro, as well as in animal models.[54] This virus is undergoing clinical evaluation in patients with glioblastoma multiforme and multiple myeloma.

Newcastle Disease Virus

Lytic strains of the avian paramyxovirus Newcastle disease virus (NDV) selectively kill cancer cells in culture and in mouse models.[55] The molecular basis of selectivity is not fully understood, but appears to be facilitated by high levels of N-myc, at least in neuroblastoma cells. Cytotoxicity is due to multiple caspase-dependent pathways of apoptosis independent of interferon signaling competence.[55,56] Several Phase I studies of intravenously infused NDV have been performed using various doses and administration schedules.[57,58] Main toxicities included moderate flu-like symptoms and mild gastrointestinal symptoms. Interestingly, a two-step intrapatient dose-escalation of the NDV strain PV701 aiming at desensitizing resulted in significant reduction of the intensity of adverse events. Patients developed only moderate levels of neutralizing antibodies, and the serum clearing of virus was not significantly different during the course of treatment.[59] Disease stabilization as well as objective tumor responses were observed in Phase I studies, in particular in patients

who had received higher doses. A complete remission was observed in a patient with glioblastoma multiforme treated with the NDV strain NDV-HUJ.[57]

Viruses Engineered to Replicate Selectively

In addition to naturally occurring viruses, many efforts have been made to engineer viruses to replicate in tumor cells selectively. Such agents kill cells through lytic mechanisms and potentially spread from one infected cell to another, amplifying the dose of the selective killing agent. Selectivity for cancer cells can be achieved by several strategies. The first uses DNA synthetic enzymes produced by proliferating tumor cells to support replication of DNA viruses that are otherwise defective. The second takes advantage of genetic defects in cancer cells that supply functions that have been specifically deleted from the oncolytic agent, and the third uses tumor-selective promoters to drive replication of conditionally replicating viruses.

Herpes Simplex Viruses

One of the first viruses designed to replicate in cancer selectively were HSVs that had been engineered so that they were unable to express viral genes necessary for DNA replication, such as thymidine kinase or ribonucleotide reductase. Proliferating cells would provide these essential functions, whereas resting normal cells would not. HSV G207 is an example of such an oncolytic virus. In addition to inactivation of a subunit of the viral ribonucleotide reductase gene, both copies of the neurovirulence gene, the gamma(1)34.5 gene, are deleted to further reduce replication in normal tissues.[60] Although direct tumor cell killing represents a major mechanism of action of these viruses, evidence from experiments in immunocompetent mouse models suggests that also a vaccination effect mediated by activated T lymphocytes contributes to the effect.[61] Phase I clinical trials of G207 and a related virus, HSV1716, have been completed and demonstrated the safety of these viruses.[62] Another related HSV mutant, NV1020, has been tested in a Phase I study in patients with hepatic metastases from colorectal cancer. The virus was administered into the hepatic artery in a Phase I study. Only mild toxicity was observed, and a decline in CEA levels was suggestive of some antitumor activity.[63] Many innovative approaches have been employed to improve the clinical value of HSV viruses, including expression of prodrug-converting enzymes to elicit bystander cells and the addition of genes encoding cytokines to boost immune recognition of tumor cells. The latter approach has been taken into the clinic in the form of the HSV mutant OncoVex(GM-CSF), a conditionally replicating HSV-1 mutant that expresses the cytokine GM-CSF. A Phase I/II trial with intratumorally injected Oncovex GM-CSF demonstrated that this agent is well tolerated.[64] Side effects included mainly fever, flu-like symptoms, and inflammation at the injection site. Clinical evidence for tumor necrosis was found; however, no detailed objective response assessments have been published at this point.

Vaccinia

Using a similar strategy, attenuated strains of another large DNA virus, vaccinia, have been created and shown to replicate selectively in cancer cells.[65] Vaccinia virus (VV) strains have several attributes that render them ideally suited for their use as oncolytic agents. First, VV vectors are derived from vaccine strains that have been used safely in millions of children worldwide for immunization against smallpox. Thus, their safety profile, genetic stability, and other pharmacological parameters are extremely well documented. Second, these viruses have several suitable biological properties: vaccinia has evolved mechanisms for stability in the bloodstream and spread to distant sites, including resistance to antibody and complement-mediated neutralization. Moreover, because of their relatively large size (about 200 nm), vaccinia virions preferentially accumulate in tumor tissues, where neovasculature has increased permeability ("leaky tumor vasculature"). The relatively large size also results in a large transgene capacity (25 to 50 kb), which allows for the expression of several therapeutic and monitoring genes. Last but not least, VV replication is promoted by EGFR/Ras pathway signaling,[66,67] which is frequently hyperactivated in cancer cells. A genetically engineered vaccinia that has shown great promise in recent clinical trials is JX-594.[68] This virus is a derivative of a smallpox-vaccine strain carrying an inactivated *TK* gene to increase tumor specificity, and two transgenes: one encoding GM-CSF to stimulate antitumor immune responses and the other β-galactosidase, as a surrogate marker for viral gene expression. JX-594 has demonstrated great success on local-regional and intravenous delivery to patients in terms of safety, cancer selectivity, oncolysis, chemosensitization, and induction of immune and antitumor responses (Figure 54-1, Table 54-3). Notably, in a Phase I clinical trial, intravenously delivered JX-594 was capable of replicating selectively in metastases from a variety of tumor types, representing a milestone in the development of oncolytic agents for systemic administration. Moreover, intravenous JX-594 therapy led to a reduction in the outgrowth of new metastases in patients over time. Seven Phase I and II clinical trials are currently ongoing to test this agent's safety and efficacy further in various solid tumors.[69,70]

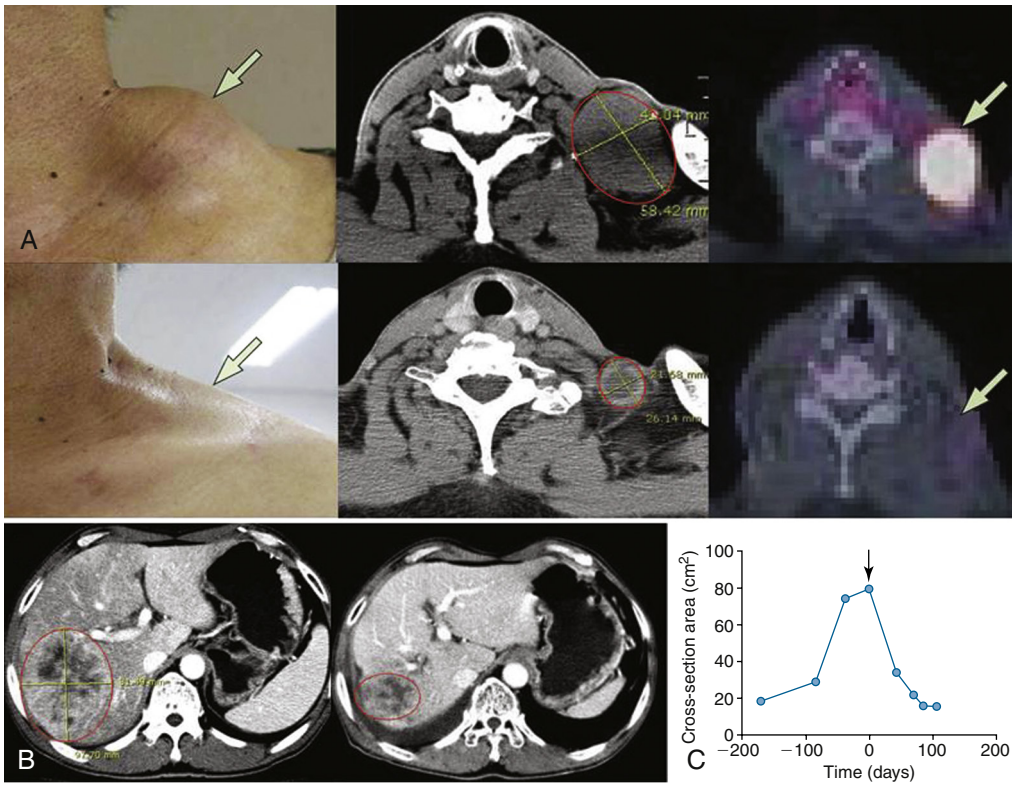


Figure 54-1 Representative tumor response to JX-594 treatment **(A)** Patient 201 with a primary hepatocellular carcinoma received direct intratumoral injections into the liver tumor, which led to a RECIST PR. Direct injection of a previously noninjected tumor in the neck, after four previous cycles of JX-594 treatment in the liver, led to RECIST and Choi response at this site as well, despite the presence of high concentrations of neutralizing antibodies to JX-594. Physical examination and CT and PET-CT scans of the metastatic tumor in the neck region, before and after induction of high-titer antibodies, illustrate this response. **(B, C)** Patient 103 with metastatic squamous cell carcinoma of the lung received six rounds of JX-594 intratumoral injections. **(B)** CT scan and **(C)** tumor cross-section areas before and after treatment. *Arrow* indicates initiation of JX-594 treatment. *CT,* Computed tomography; *PET,* positron emission tomography; *PR,* partial response; *RECIST,* Response Evaluation Criteria in Solid Tumors. *(Adapted from Park BH, Hwang T, Liu TC, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial.* Lancet Oncol. *2008;9:533-542.)*

Table 54-3 Representative Patient Response to JX-594

Patient ID	Tumor	Injected	Response	
			RECIST*	Choi criteria**
201	Primary tumor: hepatocellular carcinoma (HCC)	Yes	Partial response (PR)	+ (30% decrease in diameter)
	Secondary tumor: neck	Yes	PR	+ (57% decrease in diameter; see Figure 54-1A)
103	Primary tumor: lung squamous cell carcinoma	Yes	PR	+ (51% decrease in diameter; see Figure 54-1B, C)
	Metastasis: liver	No	Stable disease (SD)	+ (22% decrease in HU†)

Adapted from Park BH, Hwang T, Liu TC, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol.* 2008;9:533-542.
*RECIST: Response Evaluation Criteria in Solid Tumors: Partial response (PR) is a maximum diameter decrease of ≥30%, progressive disease (PD) is an increase of ≥20%, and stable disease (SD) is a change in diameter between these two cutoffs.
**Choi criteria: response (+) is maximum diameter decrease of ≥10% or density decrease of ≥15%.
†HU: Hounsfield units, also CT units: related to the composition and nature of the tissue imaged, represents the density of tissue.

Adenovirus

Whereas G207 and related viruses were engineered to take advantage of permissive conditions in cancer cells relative to quiescent cells, ONYX-015 was designed to exploit lack of functional p53 in tumor cells. ONYX-015 is an adenovirus that lacks the E1B 55K gene. This gene encodes a protein that binds p53 and targets it for degradation. In the absence of E1B 55K, ONYX-015 was expected to replicate poorly in normal cells, in which functional p53 could abort lytic,

productive replication. In contrast, cancer cells should be permissive for ONYX-015 because E1B 55k should be unnecessary in cells that lack p53.[71]

Extensive analysis of the molecular mechanisms underlying ONYX-015 replication revealed that the virus does indeed replicate selectively in tumor cells. Whereas replication of this virus is mediated through the p53 pathway, tumor selectivity is mostly based on the ability of tumor cells to complement other functions of E1B 55K unrelated to p53.[72-74] These functions relate to the ability of E1B 55K to facilitate the export of viral mRNAs and shut down host protein synthesis. Nonetheless, ONYX-015 advanced to Phase III trials in the United States, after encouraging signs of safety and efficacy. The analysis of clinical tumor specimens obtained following injection of ONYX-015 provided unambiguous evidence for its tumor selectivity. Replicating virus was found (1) in head and neck cancer following intratumoral injection, (2) in liver metastases from gastrointestinal cancer after intra-arterial injection, and (3) in lung tumors following intravenous injection.[75-77] Furthermore, objective tumor responses were observed in several clinical Phase II studies. Single-agent treatment with ONYX-015 induced objective responses in 14% of patients with recurrent head and neck cancer.[77] In a subsequent study, the virus was combined with standard chemotherapy. Only a single tumor was injected with ONYX-015, leaving a subgroup of patients with additional uninjected control lesions. Interestingly, the response rate of tumors injected with the virus was significantly greater than those of noninjected lesions, and the time to progression was significantly longer for injected tumors.[75] Further evidence of antitumor activity of ONYX-015 was found in three patients with 5-FU/leucovorin-refractory liver metastases from colorectal cancer that experienced minor responses (30% to 48% shrinkage) following intra-arterial infusion of ONYX-015 into the hepatic artery.[78]

Meanwhile, a closely related adenovirus, H101, has recently been developed by Shanghai Sunway Biotech and approved for treatment of head and neck cancer in China[3] after a clinical Phase III study demonstrated a dramatically higher tumor response rate in patients who had received H101 in combination with cisplatin and 5-fluorouracil (78.8% versus 39.6%, respectively).[79]

Delta-24 (also known as *dl*922-947 or ONYX-838) is another adenovirus mutant that targets cancer cells selectively.[80] The E1a region contains a small deletion that prevents binding to RB. As a result, this virus cannot replicate efficiently in normal cells, because RB represses E2F activity that is essential for replication (in addition to transcribing genes involved in DNA synthesis, E2F activates transcription of the viral E2 region; this is how E2F was first named and identified). In cancer cells, E2F activity is not repressed by RB, because RB itself is mutated or inactivated indirectly through loss of p16INK4a, amplification of cyclin D1, or by other means. Tumor cells therefore provide a permissive environment for replication of Delta-24. A modified version of Delta-24 has entered clinical trials for the treatment of glioblastoma.[81,82] This virus, Delta-24 RGD, has been engineered to increase infectivity by the addition of an RGD sequence in its fiber gene.[83] Likewise, adenoviruses designated KD1 and KD3 contain two small deletions in E1A that abolish its binding to pRB but leave the ability of E1A to transactivate viral genes intact. These have been shown to replicate with great efficiency in tumor cells, but fail to replicate efficiently in normal cells.[84]

A second-generation version of Delta-24 was engineered to express human p53.[85,86] Ad24-p53 more effectively killed most human cancer cell lines tested in vitro than did its parent Ad24 and had significant activity against xenografts in vivo.[85] To further improve potency of this virus, the p53 transgene was engineered so that it is resistant to degradation by Mdm2.[85] Adenoviruses have also been engineered to take advantage of unregulated TCF transcriptional activity, a characteristic of colorectal cancer cells that lack the APC tumor suppressor or contain activating mutations in beta-catenin.[87,88]

An array of creative approaches has been employed to make viruses replicate selectively based on abnormal transcription activity in cancer cells. These include viruses that use unregulated E2F activity resulting from loss of the RB tumor suppressor pathway to drive the E2F-1 promoter, the telomerase promoter, prostate-specific promoters and regulatory elements to drive proliferation in prostate cancer cells, and many others. These approaches have been reviewed recently by Fukazawa and colleagues.[89]

Challenges and Future Perspective

Preclinical Development

Gene therapeutic agents and oncolytic viruses have highly diverse physical and biological properties and act through complex molecular mechanisms, making predictions about their pharmacological and pharmacodynamic behavior in humans difficult. Preclinical model systems are therefore particularly important elements in the selection process for further clinical development.

Mouse xenograft tumor models of human cancer cell lines in nude mice allow for efficient assessment of antitumor activities of novel gene delivery and oncolytic agents in a broad variety of human tumor types. However, these models have significant limitations due to the lack of a functional immune system and differences in structure and composition

of the tumor stroma. To address these limitations, immunocompetent tumor models have been developed. Such models have been instrumental for assessing the impact of immunemodulatory genes in the genome of oncolytic adenoviruses on virus replication and antitumor effect.[90] In addition, orthotopic implantation of allografts in immunocompetent models allows for testing novel vectors in tumors growing an organotypic microenvironment that more closely resembles the situation in humans.[91-93] Despite these improved murine models, limitations remain. For example, normal and malignant mouse tissues only poorly support replication of human adenoviruses. Another example is the difference between the sequences of human and mouse cytokines, which makes the generation of mouse-specific variants of immune-modulatory agents necessary. Other species offer potentially advantageous features. For example, normal and malignant Syrian hamster cells support adenovirus replication. Using this model, intratumoral injection of an oncolytic adenovirus resulted in suppression not only of the primary tumor but also of distant metastases following virus entry into the bloodstream.[94] Similar results were obtained in cotton rats.[95] In addition, replication-competent viruses for use in canine models have been developed.[96]

Biodistribution and systemic effects of novel gene therapy and oncolytic viral agents are fundamentally different from small-molecule or antibody-based anticancer therapies and therefore difficult to predict. To address this, novel in vivo imaging strategies allowing for real-time monitoring of the effects of such agents in animals as well as in humans have been developed and will play a major role in the further development of this therapeutic approach. Distribution of nonviral and viral particles can be directly assessed by radioactive or fluorescent labeling. The biodistribution of liposomes, for example, can be followed after labeling with radioactive isotopes (e.g., ^{99m}Tc) or gadolinium by scintigraphy or magnetic resonance imaging, respectively.[97,98] A variety of strategies have been pursued to monitor viral agents. Green-fluorescent protein and firefly luciferase are transgenes that allow for the detection of cells infected with viruses carrying an expression cassette for either of these genes through the detection of fluorescent light or bioluminescence, respectively—in animal models, at least.[99,100] The use of prodrug-converting enzymes opens the possibility of using the enzyme activity for imaging purposes. The most developed approach is expression of the prodrug converting enzyme HSV-TK, which not only converts ganciclovir into cytotoxic phosphorylated derivatives but also phosphorylates uracil derivatives labeled with radioactive iodine and acylguanosines labeled with radioactive fluorine, which are then also retained within the cell and detectable by positron emission tomography (PET). This approach has been successfully used in a variety of vectors in small animals. Recently, it has also been demonstrated to be an effective imaging strategy in a pilot study in patients with liver cancer.[101] A similar approach involves the vector-mediated delivery of receptors with only limited physiologic expression, such as the dopamine D2 receptor or the somatostatin receptor subtype 2. PET imaging probes for both receptors are already clinically available for imaging of neuroendocrine tumors. A third technology has been developed using the sodium-iodide symporter, which is a transmembrane transporter protein that is physiologically predominantly expressed in the thyroid gland.[102] Ectopic expression of this protein leads to accumulation of radioactive iodine, which can be detected by radionuclide imaging using a gamma camera or PET. The potential advantage of this approach is that cytotoxic radionuclides such as ^{131}I can be used therapeutically.[103]

The complexity of the host-vector interaction and the resulting dynamics of virus and tumor cell replication are theoretically amenable to in silico modeling of virus and tumor cell population dynamics. Such mathematical models created important insights into the kinetics of human immunodeficiency virus (HIV) infection and treatment[104,105] and have been, at a theoretical level, developed for oncolytic viruses and their interaction with tumor cells and the host immune system.[106,107] Experimental validation of these models is currently being actively pursued by several laboratories.

Safety and Toxicity

The use of viruses either as vectors for the delivery of therapeutic genes or, in mutant form, as therapeutics themselves raised significant concerns not only in regard to the safety of individuals treated with such agents but also because of the potential risks for others. In particular, the occurrence of recombinant viruses that regain wild-type properties or demonstrate even greater toxicity was feared. For this reason, extensive safety studies were performed. For example, the biohazard potential of AdCMV-p53 was investigated in France in the context of clinical trials of this virus in head and neck cancer. No evidence for any environmental risk from intratumoral injection of the virus was found.[108]

Immune and Cytokine Response

A major concern with respect to the use of viral particles as therapeutic agents is the induction of neutralizing antibodies that could limit the efficacy of such agents. In the case of adenovirus this is of particular relevance, as at least 50%

of patients present with preexisting antibodies against adenovirus type 5, resulting from earlier infections.[75-78,109,110] It is therefore not surprising that almost all of the patients treated with viral agents developed high titers of neutralizing antibodies following the first administration of the virus. Preclinical studies suggest that the presence of such antibodies might reduce the efficacy of adenoviral treatments.[111] At this point, this has not been clearly demonstrated clinically. In contrast, following intra-arterial infusion of ONYX-015 into the hepatic artery or intravenous administration, the virus was cleared rapidly from the bloodstream.

Improving Tumor Killing by Improving Tumor Cell Access

Viral vectors and oncolytic agents have been engineered to improve their ability to infect cancer cells, either to increase selectivity or to increase potency. Adenoviruses have been the focus of many of these efforts. This is because it is believed that the utility of adenovirus vectors is limited because of the low expression of CAR, the high-affinity receptor that is necessary for efficient attachment to the cell membrane.[112] This is of particular concern in many advanced cancers, in which CAR levels are often low relative to normal cells or well-differentiated cancers.[113,114] Many attempts have been made to address this problem. Wickham and co-workers[115] modified the C terminus of the adenoviral fiber protein by the addition of either an RGD-containing peptide or seven lysine residues. Dmitriev and colleagues[116,117] have also shown that the incorporation of an RGD-containing peptide in the H1 loop of the fiber knob domain results in the ability of the virus to utilize an alternative receptor during the cell entry process. The modified virus was able to infect primary tumor cells and tumor cell lines more efficiently than unmodified virus.[116,118] The RGD/fiber modification was subsequently introduced into the Delta-24 virus, described above. Gu and associates have successfully redirected cell binding and uptake of an adenovirus through fibroblast growth factor receptors (FGFRs), suggesting that redirecting the native tropism of adenovirus may offer therapeutic benefit.[119]

Conclusion

Virotherapy and other gene-therapeutic approaches represent a novel class of targeted anticancer agents that is distinct from traditional treatment modalities.

The initial focus of this field was to invent and test new agents that kill cancer cells selectively, based on the genetic lesions that cause this disease. This goal has been achieved using a rich variety of viruses, vectors, genes, and approaches, in laboratory and animal model settings. Some of these approaches have been translated into clinical research projects; however, until very recently, clinical results have been disappointing in terms of increased survival or meaningful patient benefit.[120] Although most treatment regimens have shown great safety, some early gene therapeutic trials raised serious concerns in this regard—the death of a patient with ornithine transcarbamylase deficiency, and the leukemia caused by gene therapy in four severe combined immunodeficiency (SCID) patients.[121] These rare adverse effects and the initial lack of compelling clinical activity, together with the potential risk and cost of manufacturing these agents, has dampened the initial enthusiasm in this field, as well as interfered with significant investments. However, substantial advances in our understanding of cancer biology and validation of targets, as well as the promise shown by some of the very recent gene therapeutic clinical trials, have led to a paradigm shift: anticancer gene therapy is now on an upswing again.

Nonetheless, significant hurdles still exist and need to be addressed. One of the biggest remaining challenges is delivery, including delivery to the tumor site, spread within the tumor, undesired clearance by the liver, neutralizing antibodies, and complement. Another significant difficulty for many of the approaches currently in development is the lack of appropriate preclinical models.

For gene therapy strategies to be clinically successful, they likely have to be included in multimodality treatment approaches and go far beyond killing cancer cells: they need to be anti-angiogenic and immune-modulatory, and they must prevent or overcome resistance to therapy. Intelligent vector design and clever combination regimens will, it is hoped, bring us closer to achieving this goal. Recent studies have demonstrated synergistic effects of combination therapies consisting of conventional chemo- or radio- and virotherapy. Moreover, combinatorial therapies with more "modern" approaches, such as the use of nanotechnology and cellular carriers, hold great promise. For instance, polymer-coated adenoviruses have shown a significantly enhanced half-life when compared to naked viruses, and the use of appropriate cellular carriers has been demonstrated to improve delivery and induction of desirable immune responses against the tumor.

Furthermore, clinical studies will likely yield better patient benefits and increase the chance for approval if factors can be pinpointed that predict efficacy—such as tumor type, histology, and molecular signatures.

Taken together, the recent advances in cancer biology, virology, biotechnology, and nanotechnology, as well as interdisciplinary collaborations, will expedite the development of this therapeutic platform and, we can hope, improve patients' lives in the very near future.

Key References

1. Clevers H. Wnt breakers in colon cancer. *Cancer Cell*. 2004;5:5-6.
3. Garber K. China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst*. 2006;98:298-300.
10. Peng Z. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther*. 2005;16:1016-1027.
11. Zhang SW, Xiao SW, Liu CQ, et al. [Recombinant adenovirus-p53 gene therapy combined with radiotherapy for head and neck squamous-cell carcinoma]. *Zhonghua Zhong Liu Za Zhi*. 2005;27:426-428.
14. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. *Cancer Res*. 2012;72:2457-2467.
17. Kurreck J. Antisense technologies: Improvement through novel chemical modifications. *Eur J Biochem*. 2003;270:1628-1644.
18. Bedikian AY, Millward M, Pehamberger H, et al. Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. *J Clin Oncol*. 2006;24:4738-4745.
19. Dikmen ZG, Gellert GC, Jackson S, et al. In vivo inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. *Cancer Res*. 2005;65:7866-7873.
20. Devi GR. siRNA-based approaches in cancer therapy. *Cancer Gene Ther*. 2006;13:819-829.
21. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*. 2003;21:635.
23. Cerqueira NM, Pereira S, Fernandes PA, Ramos MJ. Overview of ribonucleotide reductase inhibitors: an appealing target in anti-tumour therapy. *Curr Med Chem*. 2005;12:1283-1294.
24. Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010;464:1067-1070.
25. Duarte S, Carle G, Faneca H, Lima MC, Pierrefite-Carle V. Suicide gene therapy in cancer: Where do we stand now? *Cancer Lett*. 2012;324:160-170.
26. Rainov NG, Ren H. Clinical trials with retrovirus mediated gene therapy—what have we learned? *J Neurooncol*. 2003;65:227-236.
32. Oosterhoff D, Overmeer RM, de Graaf M, et al. Adenoviral vector-mediated expression of a gene encoding secreted, EpCAM-targeted carboxylesterase-2 sensitises colon cancer spheroids to CPT-11. *Br J Cancer*. 2005;92:882-887.
33. Ottolino-Perry K, Diallo JS, Lichty BD, Bell JC, McCart JA. Intelligent design: combination therapy with oncolytic viruses. *Mol Ther*. 2010;18:251-263.
34. Opyrchal M, Allen C, Iankov I, et al. Effective radiovirotherapy for malignant gliomas by using oncolytic measles virus strains encoding the sodium iodide symporter (MV-NIS). *Hum Gene Ther*. 2012;23:419-427.
35. Immonen A, Vapalahti M, Tyynela K, et al. AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. *Mol Ther*. 2004;10:967-972.
37. Patel P, Mautner V, Searle PF, et al. Suicide gene therapy using adenovirus encoded nitroreductase and CB1954 in patients with locally relapsed prostate cancer. *J Clin Oncol (Meeting Abstracts)*. 2005;23:3091.
38. Palmer DH, Mautner V, Mirza D, et al. Virus-directed enzyme prodrug therapy: intratumoral administration of a replication-deficient adenovirus encoding nitroreductase to patients with resectable liver cancer. *J Clin Oncol*. 2004;22:1546-1552.
45. Huang TG, Savontaus MJ, Shinozaki K, Sauter BV, Woo SL. Telomerase-dependent oncolytic adenovirus for cancer treatment. *Gene Ther*. 2003;10:1241-1247.
46. Wirth T, Zender L, Schulte B, et al. A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res*. 2003;63:3181-3188.
48. Senzer N, Rosemurgy A, Javle M, et al. The PACT trial: Interim results of a randomized trial of TNFerade biologic plus chemoradiation (CRT) compared to CRT alone in locally advanced pancreatic cancer (LAPC). *J Clin Oncol (Meeting Abstracts)*. 2006;24:4102.
49. Burke JM. GM-CSF-armed, replication-competent viruses for cancer. *Cytokine Growth Factor Rev*. 2010;21:149-151.
50. Coffey MC, Strong JE, Forsyth PA, Lee PW. Reovirus therapy of tumors with activated Ras pathway. *Science*. 1998;282:1332-1334.
51. Vidal L, Pandha H, Spicer J, et al. A phase I study of reolysin given intravenously to patients with advanced malignancies. *ASCO Meeting Abstracts*. 2006;24:3064.
52. Ahmed M, Cramer SD, Lyles DS. Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. *Virology*. 2004;330:34-49.
53. Lun X, Senger DL, Alain T, et al. Effects of intravenously administered recombinant vesicular stomatitis virus (VSV(deltaM51)) on multifocal and invasive gliomas. *J Natl Cancer Inst*. 2006;98:1546-1557.
54. Phuong LK, Allen C, Peng KW, et al. Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. *Cancer Res*. 2003;63:2462-2469.
56. Elankumaran S, Rockemann D, Samal SK. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. *J Virol*. 2006;80:7522-7534.
57. Freeman AI, Zakay-Rones Z, Gomori JM, et al. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther*. 2006;13:221-228.
58. Laurie SA, Bell JC, Atkins HL, et al. A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin Cancer Res*. 2006;12:2555-2562.
59. Lorence RM, Pecora AL, Major PP, et al. Overview of phase I studies of intravenous administration of PV701, an oncolytic virus. *Curr Opin Mol Ther*. 2003;5:618-624.
62. Shen Y, Nemunaitis J. Herpes simplex virus 1 (HSV-1) for cancer treatment. *Cancer Gene Ther*. 2006;13:975-992.
63. Kemeny N, Brown K, Covey A, et al. Phase I, open-label, dose-escalating study of a genetically engineered herpes simplex virus, NV1020, in subjects with metastatic colorectal carcinoma to the liver. *Hum Gene Ther*. 2006;17:1214-1224.
64. Coffin RS, Liu B, Han Z, et al. OncoVEX: A family of oncolytic herpes simplex viruses optimised for therapeutic use. *ASCO Meeting Abstracts*. 2006;24:13139.
65. Kirn DH, Thorne SH. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer*. 2009;9:64-71.
66. Katsafanas GC, Moss B. Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *J Biol Chem*. 2004;279:52210-52217.
67. Parato KA, Breitbach CJ, Le Boeuf F, et al. The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther*. 2012;20:749-758.
68. Breitbach CJ, Burke J, Jonker D, et al. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature*. 2011;477:99-102.
70. Guo ZS, Naik A, O'Malley ME, et al. The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2. *Cancer Res*. 2005;65:9991-9998.
73. O'Shea CC, Johnson L, Bagus B, et al. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell*. 2004;6:611-623.
74. O'Shea CC, Soria C, Bagus B, McCormick F. Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell*. 2005;8:61-74.
79. Xia ZJ, Chang JH, Zhang L, et al. [Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus.]. *Ai Zheng*. 2004;23:1666-1670.
81. Jiang H, Gomez-Manzano C, Lang FF, Alemany R, Fueyo J. Oncolytic adenovirus: preclinical and clinical studies in patients with human malignant gliomas. *Curr Gene Ther*. 2009;9:422-427.
82. Alonso MM, Jiang H, Gomez-Manzano C, Fueyo J. Targeting brain tumor stem cells with oncolytic adenoviruses. *Methods Mol Biol*. 2012;797:111-125.
83. Fueyo J, Alemany R, Gomez-Manzano C, et al. Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst*. 2003;95:652-660.

85. Geoerger B, Vassal G, Opolon P, et al. Oncolytic activity of p53-expressing conditionally replicative adenovirus AdDelta24-p53 against human malignant glioma. *Cancer Res*. 2004;64:5753-5759.
86. Mitlianga PG, Sioka C, Vartholomatos G, et al. p53 enhances the Delta-24 conditionally replicative adenovirus anti-glioma effect. *Oncol Rep*. 2006;15:149-153.
89. Fukazawa T, Matsuoka J, Yamatsuji T, Maeda Y, Durbin ML, Naomoto Y. Adenovirus-mediated cancer gene therapy and virotherapy (Review). *Int J Mol Med*. 2010;25:3-10.
90. Wang Y, Hallden G, Hill R, et al. E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models. *Nat Biotechnol*. 2003;21:1328-1335.
92. Fodor I, Timiryasova T, Denes B, Yoshida J, Ruckle H, Lilly M. Vaccinia virus mediated p53 gene therapy for bladder cancer in an orthotopic murine model. *J Urol*. 2005;173:604-609.
93. Satoh T, Saika T, Ebara S, et al. Macrophages transduced with an adenoviral vector expressing interleukin 12 suppress tumor growth and metastasis in a preclinical metastatic prostate cancer model. *Cancer Res*. 2003;63:7853-7860.
94. Thomas MA, Spencer JF, La Regina MC, et al. Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Res*. 2006;66:1270-1276.
95. Toth K, Spencer JF, Tollefson AE, et al. Cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Hum Gene Ther*. 2005;16:139-146.
96. Le LP, Rivera AA, Glasgow JN, et al. Infectivity enhancement for adenoviral transduction of canine osteosarcoma cells. *Gene Ther*. 2006;13:389-399.
97. Bao A, Goins B, Klipper R, Negrete G, Mahindaratne M, Phillips WT. A novel liposome radiolabeling method using ^{99m}Tc-"SNS/S" complexes: in vitro and in vivo evaluation. *J Pharm Sci*. 2003;92:1893-1904.
98. Leclercq F, Cohen-Ohana M, Mignet N, et al. Design, synthesis, and evaluation of gadolinium cationic lipids as tools for biodistribution studies of gene delivery complexes. *Bioconjug Chem*. 2003;14:112-119.
99. Adusumilli PS, Stiles BM, Chan MK, et al. Real-time diagnostic imaging of tumors and metastases by use of a replication-competent herpes vector to facilitate minimally invasive oncological surgery. *FASEB J*. 2006;20:726-728.
101. Penuelas I, Mazzolini G, Boan JF, et al. Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology*. 2005;128:1787-1795.
102. Dwyer RM, Schatz SM, Bergert ER, et al. A preclinical large animal model of adenovirus-mediated expression of the sodium-iodide symporter for radioiodide imaging and therapy of locally recurrent prostate cancer. *Mol Ther*. 2005;12:835-841.
103. Dwyer RM, Bergert ER, O'Connor MK, Gendler SJ, Morris JC. Sodium iodide symporter-mediated radioiodide imaging and therapy of ovarian tumor xenografts in mice. *Gene Ther*. 2006;13:60-66.
107. Wein LM, Wu JT, Kirn DH. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res*. 2003;63:1317-1324.
114. Korn WM, Macal M, Christian C, et al. Expression of the coxsackievirus- and adenovirus receptor in gastrointestinal cancer correlates with tumor differentiation. *Cancer Gene Ther*. 2006;13:792-797.
120. Liu TC, Galanis E, Kirn D. Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol*. 2007;4:101-117.
121. Teichler Zallen D. US gene therapy in crisis. *Trends Genet*. 2000;16:272-275.
122. Lorence RM, Roberts MS, O'Neil JD, et al. Phase 1 clinical experience using intravenous administration of PV701, an oncolytic Newcastle disease virus. *Curr Cancer Drug Targets*. 2007;7:157-167.
123. Geletneky K, Huesing J, Rommelaere J, et al. Phase I/IIa study of intratumoral/intracerebral or intravenous/intracerebral administration of Parvovirus H-1 (ParvOryx) in patients with progressive primary or recurrent glioblastoma multiforme: ParvOryx01 protocol. *BMC Cancer*. 2012;12:99.
124. Rommelaere J, Geletneky K, Angelova AL, et al. Oncolytic parvoviruses as cancer therapeutics. *Cytokine Growth Factor Rev*. 2010;21:185-195.
126. Norman KL, Lee PW. Not all viruses are bad guys: the case for reovirus in cancer therapy. *Drug Discov Today*. 2005;10:847-855.
127. Ramesh N, Ge Y, Ennist DL, et al. CG0070, a conditionally replicating granulocyte macrophage colony-stimulating factor—armed oncolytic adenovirus for the treatment of bladder cancer. *Clin Cancer Res*. 2006;12:305-313.
128. Liu BL, Robinson M, Han ZQ, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther*. 2003;10:292-303.
129. Sivendran S, Pan M, Kaufman HL, Saenger Y. Herpes simplex virus oncolytic vaccine therapy in melanoma. *Expert Opin Biol Ther*. 2010;10:1145-1153.
130. Guse K, Cerullo V, Hemminki A. Oncolytic vaccinia virus for the treatment of cancer. *Expert Opin Biol Ther*. 2011;11:595-608.
131. Zhang Q, Yu YA, Wang E, et al. Eradication of solid human breast tumors in nude mice with an intravenously injected light-emitting oncolytic vaccinia virus. *Cancer Res*. 2007;67:10038-10046.
132. Heo J, Breitbach CJ, Moon A, et al. Sequential therapy with JX-594, a targeted oncolytic poxvirus, followed by sorafenib in hepatocellular carcinoma: preclinical and clinical demonstration of combination efficacy. *Mol Ther*. 2011;19:1170-1179.

This page intentionally left blank

References

1. Clevers H. Wnt breakers in colon cancer. *Cancer Cell*. 2004;5:5-6.
2. Kaye FJ. RB and cyclin dependent kinase pathways: defining a distinction between RB and p16 loss in lung cancer. *Oncogene*. 2002;21:6908-6914.
3. Garber K. China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst*. 2006;98:298-300.
4. Zhang WW, Alemany R, Wang J, Koch PE, Ordonez NG, Roth JA. Safety evaluation of Ad5CMV-p53 in vitro and in vivo. *Hum Gene Ther*. 1995;6:155-164.
5. Barak Y, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild type p53 activity. *EMBO J*. 1993;12:461-468.
6. Xu M, Kumar D, Srinivas S, et al. Parenteral gene therapy with p53 inhibits human breast tumors in vivo through a bystander mechanism without evidence of toxicity. *Hum Gene Ther*. 1997;8:177-185.
7. Reed JC, Miyashita T, Krajewski S, et al. Bcl-2 family proteins and the regulation of programmed cell death in leukemia and lymphoma. *Cancer Treat Res*. 1996;84:31-72.
8. Molinier-Frenkel V, Le Boulaire C, Le Gal FA, et al. Longitudinal follow-up of cellular and humoral immunity induced by recombinant adenovirus-mediated gene therapy in cancer patients. *Hum Gene Ther*. 2000;11:1911-1920.
9. Yen N, Ioannides CG, Xu K, et al. Cellular and humoral immune responses to adenovirus and p53 protein antigens in patients following intratumoral injection of an adenovirus vector expressing wild-type. P53 (Ad-p53). *Cancer Gene Ther*. 2000;7:530-536.
10. Peng Z. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther*. 2005;16:1016-1027.
11. Zhang SW, Xiao SW, Liu CQ, et al. [Recombinant adenovirus-p53 gene therapy combined with radiotherapy for head and neck squamous-cell carcinoma]. *Zhonghua Zhong Liu Za Zhi*. 2005;27:426-428.
12. Peng KW, TenEyck CJ, Galanis E, Kalli KR, Hartmann LC, Russell SJ. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res*. 2002;62:4656-4662.
13. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell*. 1988;53:549-554.
14. Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. *Cancer Res*. 2012;72:2457-2467.
15. Fisher GH, Wellen SL, Klimstra D, et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev*. 2001;15:3249-3262.
16. Crooke RM. In vitro toxicology and pharmacokinetics of antisense oligonucleotides. *Anticancer Drug Des*. 1991;6:609-646.
17. Kurreck J. Antisense technologies: Improvement through novel chemical modifications. *Eur J Biochem*. 2003;270:1628-1644.
18. Bedikian AY, Millward M, Pehamberger H, et al. Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. *J Clin Oncol*. 2006;24:4738-4745.
19. Dikmen ZG, Gellert GC, Jackson S, et al. In vivo inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. *Cancer Res*. 2005;65:7866-7873.
20. Devi GR. siRNA-based approaches in cancer therapy. *Cancer Gene Ther*. 2006;13:819-829.
21. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*. 2003;21:635.
22. Gatter KC, Sunderland CA, Skinner A, Sanders MK, Mason DY. An immunoelectron microscopic study of human placenta using three monoclonal antibodies. *Placenta*. 1983;4:197-205.
23. Cerqueira NM, Pereira S, Fernandes PA, Ramos MJ. Overview of ribonucleotide reductase inhibitors: an appealing target in anti-tumour therapy. *Curr Med Chem*. 2005;12:1283-1294.
24. Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010;464:1067-1070.
25. Duarte S, Carle G, Faneca H, Lima MC, Pierrefite-Carle V. Suicide gene therapy in cancer: Where do we stand now? *Cancer Lett*. 2012;324:160-170.
26. Rainov NG, Ren H. Clinical trials with retrovirus mediated gene therapy—what have we learned? *J Neurooncol*. 2003;65:227-236.
27. Freeman SM, Abboud CN, Whartenby KA, et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res*. 1993;53:5274-5283.
28. Ram Z, Culver KW, Walbridge S, Blaese RM, Oldfield EH. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res*. 1993;53:83-88.
29. Ram Z, Culver KW, Oshiro EM, et al. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med*. 1997;3:1354-1361.
30. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther*. 2000;11:2389-2401.
31. Harsh GR, Deisboeck TS, Louis DN, et al. Thymidine kinase activation of ganciclovir in recurrent malignant gliomas: a gene-marking and neuropathological study. *J Neurosurg*. 2000;92:804-811.
32. Oosterhoff D, Overmeer RM, de Graaf M, et al. Adenoviral vector-mediated expression of a gene encoding secreted, EpCAM-targeted carboxylesterase-2 sensitises colon cancer spheroids to CPT-11. *Br J Cancer*. 2005;92:882-887.
33. Ottolino-Perry K, Diallo JS, Lichty BD, Bell JC, McCart JA. Intelligent design: combination therapy with oncolytic viruses. *Mol Ther*. 2010;18:251-263.
34. Opyrchal M, Allen C, Iankov I, et al. Effective radiovirotherapy for malignant gliomas by using oncolytic measles virus strains encoding the sodium iodide symporter (MV-NIS). *Hum Gene Ther*. 2012;23:419-427.
35. Immonen A, Vapalahti M, Tyynela K, et al. AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. *Mol Ther*. 2004;10:967-972.
36. Djeha AH, Hulme A, Dexter MT, et al. Expression of Escherichia coli B nitroreductase in established human tumor xenografts in mice results in potent antitumoral and bystander effects upon systemic administration of the prodrug CB1954. *Cancer Gene Ther*. 2000;7:721-731.
37. Patel P, Mautner V, Searle PF, et al. Suicide gene therapy using adenovirus encoded nitroreductase and CB1954 in patients with locally relapsed prostate cancer. *J Clin Oncol (Meeting Abstracts)*. 2005;23:3091.
38. Palmer DH, Mautner V, Mirza D, et al. Virus-directed enzyme prodrug therapy: intratumoral administration of a replication-deficient adenovirus encoding nitroreductase to patients with resectable liver cancer. *J Clin Oncol*. 2004;22:1546-1552.
39. Chung-Faye G, Palmer D, Anderson D, et al. Virus-directed, enzyme prodrug therapy with nitroimidazole reductase: a phase I and pharmacokinetic study of its prodrug, CB1954. *Clin Cancer Res*. 2001;7:2662-2668.
40. Xie X, Zhao X, Liu Y, et al. Adenovirus-mediated tissue-targeted expression of a caspase-9-based artificial death switch for the treatment of prostate cancer. *Cancer Res*. 2001;61:6795-6804.
41. Gotoh A, Ko SC, Shirakawa T, et al. Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J Urol*. 1998;160:220-229.
42. Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res*. 1999;59:1498-1504.
43. Siders WM, Halloran PJ, Fenton RG. Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res*. 1996;56:5638-5646.
44. Anderson LM, Krotz S, Weitzman SA, Thimmapaya B. Breast cancer-specific expression of the Candida albicans cytosine deaminase gene using a transcriptional targeting approach. *Cancer Gene Ther*. 2000;7:845-852.
45. Huang TG, Savontaus MJ, Shinozaki K, Sauter BV, Woo SL. Telomerase-dependent oncolytic adenovirus for cancer treatment. *Gene Ther*. 2003;10:1241-1247.
46. Wirth T, Zender L, Schulte B, et al. A telomerase-dependent conditionally replicating adenovirus for selective treatment of cancer. *Cancer Res*. 2003;63:3181-3188.
47. Hallahan DE, Mauceri HJ, Seung LP, et al. Spatial and temporal control of gene therapy using ionizing radiation. *Nat Med*. 1995;1:786-791.

48. Senzer N, Rosemurgy A, Javle M, et al. The PACT trial: Interim results of a randomized trial of TNFerade biologic plus chemoradiation (CRT) compared to CRT alone in locally advanced pancreatic cancer (LAPC). *J Clin Oncol (Meeting Abstracts)*. 2006;24:4102.
49. Burke JM. GM-CSF-armed, replication-competent viruses for cancer. *Cytokine Growth Factor Rev*. 2010;21:149-151.
50. Coffey MC, Strong JE, Forsyth PA, Lee PW. Reovirus therapy of tumors with activated Ras pathway. *Science*. 1998;282:1332-1334.
51. Vidal L, Pandha H, Spicer J, et al. A phase I study of reolysin given intravenously to patients with advanced malignancies. *ASCO Meeting Abstracts*. 2006;24:3064.
52. Ahmed M, Cramer SD, Lyles DS. Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. *Virology*. 2004;330:34-49.
53. Lun X, Senger DL, Alain T, et al. Effects of intravenously administered recombinant vesicular stomatitis virus (VSV(deltaM51)) on multifocal and invasive gliomas. *J Natl Cancer Inst*. 2006;98:1546-1557.
54. Phuong LK, Allen C, Peng KW, et al. Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. *Cancer Res*. 2003;63:2462-2469.
55. Lorence RM, Reichard KW, Katubig BB, et al. Complete regression of human neuroblastoma xenografts in athymic mice after local Newcastle disease virus therapy. *J Natl Cancer Inst*. 1994;86:1228-1233.
56. Elankumaran S, Rockemann D, Samal SK. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. *J Virol*. 2006;80:7522-7534.
57. Freeman AI, Zakay-Rones Z, Gomori JM, et al. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther*. 2006;13:221-228.
58. Laurie SA, Bell JC, Atkins HL, et al. A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin Cancer Res*. 2006;12:2555-2562.
59. Lorence RM, Pecora AL, Major PP, et al. Overview of phase I studies of intravenous administration of PV701, an oncolytic virus. *Curr Opin Mol Ther*. 2003;5:618-624.
60. Mineta T, Rabkin SD, Yazaki T, Hunter WD, Martuza RL. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat Med*. 1995;1:938-943.
61. Endo T, Toda M, Watanabe M, et al. In situ cancer vaccination with a replication-conditional HSV for the treatment of liver metastasis of colon cancer. *Cancer Gene Ther*. 2002;9:142-148.
62. Shen Y, Nemunaitis J. Herpes simplex virus 1 (HSV-1) for cancer treatment. *Cancer Gene Ther*. 2006;13:975-992.
63. Kemeny N, Brown K, Covey A, et al. Phase I, open-label, dose-escalating study of a genetically engineered herpes simplex virus, NV1020, in subjects with metastatic colorectal carcinoma to the liver. *Hum Gene Ther*. 2006;17:1214-1224.
64. Coffin RS, Liu B, Han Z, et al. OncoVEX: A family of oncolytic herpes simplex viruses optimised for therapeutic use. *ASCO Meeting Abstracts*. 2006;24:13139.
65. Kirn DH, Thorne SH. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer*. 2009;9:64-71.
66. Katsafanas GC, Moss B. Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *J Biol Chem*. 2004;279:52210-52217.
67. Parato KA, Breitbach CJ, Le Boeuf F, et al. The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther*. 2012;20:749-758.
68. Breitbach CJ, Burke J, Jonker D, et al. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature*. 2011;477:99-102.
69. Puhlmann M, Gnant M, Brown CK, Alexander HR, Bartlett DL. Thymidine kinase-deleted vaccinia virus expressing purine nucleoside phosphorylase as a vector for tumor-directed gene therapy. *Hum Gene Ther*. 1999;10:649-657.
70. Guo ZS, Naik A, O'Malley ME, et al. The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2. *Cancer Res*. 2005;65:9991-9998.
71. Bischoff JR, Kirn DH, Williams A, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science*. 1996;274:373-376.
72. Ries SJ, Brandts CH, Chung AS, et al. Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). *Nat Med*. 2000;6:1128-1133.
73. O'Shea CC, Johnson L, Bagus B, et al. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell*. 2004;6:611-623.
74. O'Shea CC, Soria C, Bagus B, McCormick F. Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy. *Cancer Cell*. 2005;8:61-74.
75. Khuri FR, Nemunaitis J, Ganly I, et al. A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nat Med*. 2000;6:879-885.
76. Reid T, Galanis E, Abbruzzese J, et al. Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: a phase I trial. *Gene Ther*. 2001;8:1618-1626.
77. Nemunaitis J, Cunningham C, Buchanan A, et al. Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. *Gene Ther*. 2001;8:746-759.
78. Reid T, Galanis E, Abbruzzese J, et al. Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. *Cancer Res*. 2002;62:6070-6079.
79. Xia ZJ, Chang JH, Zhang L, et al. [Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus.]. *Ai Zheng*. 2004;23:1666-1670.
80. Fueyo J, Gomez-Manzano C, Alemany R, et al. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene*. 2000;19:2-12.
81. Jiang H, Gomez-Manzano C, Lang FF, Alemany R, Fueyo J. Oncolytic adenovirus: preclinical and clinical studies in patients with human malignant gliomas. *Curr Gene Ther*. 2009;9:422-427.
82. Alonso MM, Jiang H, Gomez-Manzano C, Fueyo J. Targeting brain tumor stem cells with oncolytic adenoviruses. *Methods Mol Biol*. 2012;797:111-125.
83. Fueyo J, Alemany R, Gomez-Manzano C, et al. Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst*. 2003;95:652-660.
84. Doronin K, Toth K, Kuppuswamy M, Ward P, Tollefson AE, Wold WS. Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J Virol*. 2000;74:6147-6155.
85. Geoerger B, Vassal G, Opolon P, et al. Oncolytic activity of p53-expressing conditionally replicative adenovirus AdDelta24-p53 against human malignant glioma. *Cancer Res*. 2004;64:5753-5759.
86. Mitlianga PG, Sioka C, Vartholomatos G, et al. p53 enhances the Delta-24 conditionally replicative adenovirus anti-glioma effect. *Oncol Rep*. 2006;15:149-153.
87. Brunori M, Malerba M, Kashiwazaki H, Iggo R. Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. *J Virol*. 2001;75:2857-2865.
88. Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. *Gene Ther*. 2002;9:270-281.
89. Fukazawa T, Matsuoka J, Yamatsuji T, Maeda Y, Durbin ML, Naomoto Y. Adenovirus-mediated cancer gene therapy and virotherapy (Review). *Int J Mol Med*. 2010;25:3-10.
90. Wang Y, Hallden G, Hill R, et al. E3 gene manipulations affect oncolytic adenovirus activity in immunocompetent tumor models. *Nat Biotechnol*. 2003;21:1328-1335.
91. Eastham JA, Chen SH, Sehgal I, et al. Prostate cancer gene therapy: herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum Gene Ther*. 1996;7:515-523.
92. Fodor I, Timiryasova T, Denes B, Yoshida J, Ruckle H, Lilly M. Vaccinia virus mediated p53 gene therapy for bladder cancer in an orthotopic murine model. *J Urol*. 2005;173:604-609.
93. Satoh T, Saika T, Ebara S, et al. Macrophages transduced with an adenoviral vector expressing interleukin 12 suppress tumor growth and metastasis in a preclinical metastatic prostate cancer model. *Cancer Res*. 2003;63:7853-7860.

94. Thomas MA, Spencer JF, La Regina MC, et al. Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Res*. 2006;66:1270-1276.
95. Toth K, Spencer JF, Tollefson AE, et al. Cotton rat tumor model for the evaluation of oncolytic adenoviruses. *Hum Gene Ther*. 2005;16:139-146.
96. Le LP, Rivera AA, Glasgow JN, et al. Infectivity enhancement for adenoviral transduction of canine osteosarcoma cells. *Gene Ther*. 2006;13:389-399.
97. Bao A, Goins B, Klipper R, Negrete G, Mahindaratne M, Phillips WT. A novel liposome radiolabeling method using ^{99m}Tc-"SNS/S" complexes: in vitro and in vivo evaluation. *J Pharm Sci*. 2003;92:1893-1904.
98. Leclercq F, Cohen-Ohana M, Mignet N, et al. Design, synthesis, and evaluation of gadolinium cationic lipids as tools for biodistribution studies of gene delivery complexes. *Bioconjug Chem*. 2003;14:112-119.
99. Adusumilli PS, Stiles BM, Chan MK, et al. Real-time diagnostic imaging of tumors and metastases by use of a replication-competent herpes vector to facilitate minimally invasive oncological surgery. *FASEB J*. 2006;20:726-728.
100. Iyer M, Wu L, Carey M, Wang Y, Smallwood A, Gambhir SS. Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. *Proc Natl Acad Sci U S A*. 2001;98:14595-14600.
101. Penuelas I, Mazzolini G, Boan JF, et al. Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology*. 2005;128:1787-1795.
102. Dwyer RM, Schatz SM, Bergert ER, et al. A preclinical large animal model of adenovirus-mediated expression of the sodium-iodide symporter for radioiodide imaging and therapy of locally recurrent prostate cancer. *Mol Ther*. 2005;12:835-841.
103. Dwyer RM, Bergert ER, O'Connor MK, Gendler SJ, Morris JC. Sodium iodide symporter-mediated radioiodide imaging and therapy of ovarian tumor xenografts in mice. *Gene Ther*. 2006;13:60-66.
104. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature*. 1995;373:123-126.
105. Wei X, Ghosh SK, Taylor ME, et al. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*. 1995;373:117-122.
106. Wodarz D. Viruses as antitumor weapons: defining conditions for tumor remission. *Cancer Res*. 2001;61:3501-3507.
107. Wein LM, Wu JT, Kirn DH. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res*. 2003;63:1317-1324.
108. Yver A, Gautier E, Saulnier P, Walsh V, Duprey S, Escudier B. Clinical biosafety, distribution, genetic stability and potential for transmission of adenoviral vector for gene transfer (therapy): data from 190 subjects treated intra-tumorally with RPR/INGN 201 (Ad5CMV-p53). *Proc Am Soc Clin Oncol*. 2001;20:2001 (abstr 1046).
109. Ganly I, Eckhardt SG, Rodriguez GI, et al. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res*. 2000;6:798-806.
110. Nemunaitis J, Ganly I, Khuri F, et al. Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B-55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. *Cancer Res*. 2000;60:6359-6366.
111. Chen Y, Yu DC, Charlton D, Henderson DR. Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy. *Hum Gene Ther*. 2000;11:1553-1567.
112. Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*. 1997;275:1320-1323.
113. Rauen KA, Sudilovsky D, Le JL, et al. Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: potential relevance to gene therapy. *Cancer Res*. 2002;62:3812-3818.
114. Korn WM, Macal M, Christian C, et al. Expression of the coxsackievirus- and adenovirus receptor in gastrointestinal cancer correlates with tumor differentiation. *Cancer Gene Ther*. 2006;13:792-797.
115. Wickham TJ, Tzeng E, Shears 2nd LL, et al. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J Virol*. 1997;71:8221-8229.
116. Dmitriev I, Krasnykh V, Miller CR, et al. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol*. 1998;72:9706-9713.
117. Krasnykh V, Dmitriev I, Mikheeva G, Miller CR, Belousova N, Curiel DT. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J Virol*. 1998;72:1844-1852.
118. Kasono K, Blackwell JL, Douglas JT, et al. Selective gene delivery to head and neck cancer cells via an integrin targeted adenoviral vector. *Clin Cancer Res*. 1999;5:2571-2579.
119. Chandler LA, Doukas J, Gonzalez AM, et al. FGF2-Targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. *Mol Ther*. 2000;2:153-160.
120. Liu TC, Galanis E, Kirn D. Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol*. 2007;4:101-117.
121. Teichler Zallen D. US gene therapy in crisis. *Trends Genet*. 2000;16:272-275.
122. Lorence RM, Roberts MS, O'Neil JD, et al. Phase 1 clinical experience using intravenous administration of PV701, an oncolytic Newcastle disease virus. *Curr Cancer Drug Targets*. 2007;7:157-167.
123. Geletneky K, Huesing J, Rommelaere J, et al. Phase I/IIa study of intratumoral/intracerebral or intravenous/intracerebral administration of Parvovirus H-1 (ParvOryx) in patients with progressive primary or recurrent glioblastoma multiforme: ParvOryx01 protocol. *BMC Cancer*. 2012;12:99.
124. Rommelaere J, Geletneky K, Angelova AL, et al. Oncolytic parvoviruses as cancer therapeutics. *Cytokine Growth Factor Rev*. 2010;21:185-195.
125. Strong JE, Lee PW. The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection. *J Virol*. 1996;70:612-616.
126. Norman KL, Lee PW. Not all viruses are bad guys: the case for reovirus in cancer therapy. *Drug Discov Today*. 2005;10:847-855.
127. Ramesh N, Ge Y, Ennist DL, et al. CG0070, a conditionally replicating granulocyte macrophage colony-stimulating factor—armed oncolytic adenovirus for the treatment of bladder cancer. *Clin Cancer Res*. 2006;12:305-313.
128. Liu BL, Robinson M, Han ZQ, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther*. 2003;10:292-303.
129. Sivendran S, Pan M, Kaufman HL, Saenger Y. Herpes simplex virus oncolytic vaccine therapy in melanoma. *Expert Opin Biol Ther*. 2010;10:1145-1153.
130. Guse K, Cerullo V, Hemminki A. Oncolytic vaccinia virus for the treatment of cancer. *Expert Opin Biol Ther*. 2011;11:595-608.
131. Zhang Q, Yu YA, Wang E, et al. Eradication of solid human breast tumors in nude mice with an intravenously injected light-emitting oncolytic vaccinia virus. *Cancer Res*. 2007;67:10038-10046.
132. Heo J, Breitbach CJ, Moon A, et al. Sequential therapy with JX-594, a targeted oncolytic poxvirus, followed by sorafenib in hepatocellular carcinoma: preclinical and clinical demonstration of combination efficacy. *Mol Ther*. 2011;19:1170-1179.

George Adrian Calin and Carlo Maria Croce

55

RNA as a Therapeutic Molecule

Cancer represents the most complex genetic disease. Despite decades of investigation and the expenditure of vast resources, mortality from cancer has not decreased significantly worldwide, particularly in developed countries.[1] Many explanations have been offered, but the basic point is that we do not yet deeply understand the mechanisms by which tumorigenesis occurs. A decade ago, a new layer of genetic complexity was discovered in malignant cells by the addition of noncoding RNAs (ncRNAs; RNAs that do not code for proteins) to the list of cancer genes, and exploiting this research could provide a great treatment opportunity. Globally, during the most recent 5 years for which there are data available (2004 to 2008), overall cancer incidence rates declined slightly in men (by 0.6% per year) and were stable in women, while cancer death rates decreased only by 1.8% per year in men and by 1.6% per year in women.[1] This was largely because of the advances in early detection of breast cancer and the reduction in tobacco use over the past four decades; the therapeutic advances were less significant. For example, the oligoantisense strategy was considered for many years as an optimal alternative for developing drugs to inhibit the proteins overexpressed in cancer cells (for a review, see Reference 2). This is the case with the BCL2 antisense oligonucleotide (ASO[3,4]), but until now no other ASO agents had shown consistent and reproducible beneficial effects in clinical trials. Various publications in the past decade reported clinical studies regarding trials in Phase II (to determine whether a new treatment works) or III (to study whether a new treatment is better than standard treatment) performed on more than 1000 patients using aprinocarsen (Affinitak, LY 900003, ISIS 3512; Isis Pharmaceuticals, Carlsbad, Calif[5-10]). This is a 20-mer oligonucleotide acting as an ASO that binds to the 3′ untranslated region (3′-UTR) of human messenger RNA (mRNA) for protein kinase Cα (*PKCa*). It acts by forming an mRNA-ASO duplex through Watson-Crick binding, and it leads to RNAse-H–mediated cleavage of the *PKCa* mRNA. In all trials but one, no significant effects were identified in patients with advanced non–small-cell lung cancer, metastatic colorectal cancer, or relapsed low-grade non-Hodgkin lymphoma. Therefore, there are major reasons for developing new therapeutic modalities to cure cancer.

Cancer as a Genetic Disease of Protein-Coding Genes and Noncoding RNAs

Why the need for RNAs as therapeutic molecules? One of the most unexpected and fascinating discoveries in the past few years in molecular oncology is that in a specific tumor, abnormalities in both protein-coding genes (PCGs) and ncRNAs can be identified, and the interplay between them is causally involved in the initiation and progression of human cancers.[11-13] The "classic" molecular oncology dogma was that cancer is a genetic disease involving tumor suppressor and oncogenic proteins. Recent findings that small noncoding RNAs called microRNAs (miRNAs) are involved in the pathogenesis of most cancers reveal a new layer of complexity in the molecular architecture of human cancers (Table 55-1).

MicroRNAs represent a new class of small ncRNAs able to regulate gene expression.[14,15] MicroRNAs are distinct from, but related to, small interfering RNAs (siRNAs), which have been identified in a variety of organisms (for reviews, see References 15 and 16). These small 19- to 24-nucleotide (nt) RNAs are transcribed as long primary transcripts of several kilobases (kb) in length, named pri-miRNA. Pri-miRNAs are processed in the nucleus into precursor hairpin RNAs (70 to 100 nt in length) named pre-miRNA by the double-stranded RNA-specific ribonuclease Drosha.[17] The hairpin RNAs are transported to the cytoplasm, via an exportin-5–dependent mechanism, where they are digested by a second double-stranded specific ribonuclease named Dicer. In animals, single-stranded miRNAs bind specific mRNA through a low complementary binding site located in the target mRNA, mainly at their 3′ UTR.[14] By a mechanism that is not fully characterized, the bound mRNA remains untranslated, resulting in reduced levels of

Table 55-1 MicroRNAs as Oncogenes and Tumor Suppressors (Main Examples)

Human microRNA (location)	Putative Function/Involved Pathways	Deregulation in Tumors	Putative Functions and Targets	Molecular Regulation	Diagnostic and Prognostic Markers
let-7 family (various)	*Anti-tumorigenic:* Self-sufficiency in growth signals Insensitivity to antigrowth signals Angiogenesis	Downregulation in lung, breast, gastric, ovary, prostate, and colon cancers, CLL, and leiomyomas Downregulation of *miR-98* in head and neck cancer cells Point mutation in the *let-7e* precursor sequence affects maturation	*Molecular mechanism:* Represses cell proliferation/growth *let-7f* promotes angiogenesis *Targets:* CCND1, CDC25a, CDK6, CRD-BP, HOXA9, IMP-1, MYC, RAS, TLR4	*Regulation:* MYCN positively regulates *let-7b* transcription PPARalpha inhibits *let-7c* transcription Notch pathway regulates *let-7a* *mmu-let-7a* is highly edited after transcription LIN-28 regulates the maturation of *let-7a*	*Poor prognosis:* *let-7a-2* low expression (lung and ovarian cancer patients) *let-7b* discriminates high-risk uveal melanomas *Drug resistance:* *let-7i* affects chemotherapy potency *Therapy:* Intranasal delivery of *let-7a* adenovirus reduces growth of Ras-induced lung tumors in mice
	Oncogenic: Self-sufficiency in growth signals Apoptosis	Hypomethylation of *let-7a-3* in lung adenocarcinomas Overexpression in AML	*Molecular mechanism:* *let-7a* represses NF2 and decreases chemotherapy-induced apoptosis in vitro	*Regulation:* IL-6 dependent STAT-3 survival signaling positively regulates *let-7a*	
miR-16-1/15a cluster (13q14.3, intron 4 ncRNA *DLEU2*)	*Anti-tumorigenic:* Self-sufficiency in growth signals Evasion of apoptosis	Downregulation in CLL, DLBCLs, multiple myeloma, pituitary adenoma, prostate, and pancreatic cancers Germline mutations in B-CLL patients and NZB mouse strain	*Molecular mechanism:* Induce apoptosis in leukemia cells *miR-16* regulates cell cycle by downregulation of G0/G1 proteins *Targets:* ACVR2A (*X. tropicalis*), BCL2, CARD10, CCND1, CDK6, CDC27, CGI-38, DMTF1, MCL1, NGN2, VEGF, WNT3A	*Regulation:* Wnt/βcatenin pathway negative regulates *xtr-miR-15a/16*	*Poor prognosis:* *miR-15a* and *miR-16* higher expression in de novo aggressive CLL *Drug resistance:* *miR-16* affects chemotherapy potency and modulates sensitivity to vincristine in gastric cancer cell lines
miR-21 (17q23.1, 3′UTR TMEM49)	*Oncogenic:* Self-sufficiency in growth signals Evasion of apoptosis Invasion and metastasis	Overexpression in glioblastomas; breast, lung, prostate, colon, stomach, esophageal, and cervical carcinomas; uterine leiomyosarcoma; and DLBCL	*Molecular mechanism:* *miR-21* knockdown induces apoptosis in glioblastoma cells *miR-21* induces invasion and metastasis in colorectal cancers *Targets:* BCL2, MASPIN, PDCD4, PTEN, TPM1, RECK	*Regulation:* STAT3 regulates *miR-21* at the transcriptional level REST negatively regulates mir-21 in mouse ES TGFβ and BMP promote mir-21 maturation in human vascular smooth muscle cells AP-1 induces mir-21 in response to RAS activation in thyroid cells	*Poor prognosis:* *miR-21* high expression (in colon and breast cancer) *Good prognosis:* *miR-21* high expression in de novo DLBCL *Drug resistance:* *miR-21* affects chemotherapy potency in NCI60 cells
miR-155 (21q21.3, exon 3 ncRNA BIC)	*Oncogenic:* Evasion of apoptosis	Overexpression in pediatric BL, Hodgkin's disease, primary mediastinal lymphomas, and DLBCL, as well as in breast, lung, colon, and pancreatic cancers	*Molecular mechanism:* Pre–B-cell proliferation and lymphoblastic leukemia/high-grade lymphoma in *miR-155* transgenic mice *Targets:* AGTR1, AID, TP53INP1		*Poor prognosis:* *miR-155* high expression (in lung cancer, DLCBL, and aggressive CLL)

AML, Acute myeloid leukemia; *BL*, Burkitt's lymphoma; *CLL*, chronic lymphocytic leukemia; *DLBCL*, diffuse large B-cell lymphoma; *ES*, Ewing sarcoma.

the corresponding protein—and/or the bound mRNA can be degraded, causing a decrease in the transcript and consequently, in the corresponding protein. The role of miRNAs was proven to be important in essential biologic processes for the eukaryotic cell such as pancreatic cell insulin secretion (*miR-375*), adipocyte development (*miR-375*), cell proliferation control (*miR'125b* and Iet-7), brain patterning (*miR-430*), hematopoietic B-lymphocyte lineage fate (*miR-181*), or B-lymphocyte survival (*miR-15a* and *miR-16-1*).[12,13]

MicroRNAs were found to be involved in the pathophysiology of all types of analyzed human cancers.[18] Among the new paradigms of molecular oncology are the following:

1. Several genome-wide profiling techniques (for review, see References 13, 19, and 20), such as oligonucleotide miRNA microarray, bead-based flow cytometric technique, and quantitative reverse-transcriptase-polymerase chain reaction (qRT-PCR) for precursor and active miRNA or the miRAGE (serial analyses of gene expression for miRNAs), were performed on various cancer histotypes, including chronic lymphocytic leukemia (CLL), breast cancer, glioblastoma, thyroid papillary carcinoma, hepatocellular carcinoma, lung cancer, colon cancer, and endocrine and exocrine pancreatic tumors. From these studies it has become clear that in cancer cells the main alteration of the microRNome (defined as the full complement of microRNAs present in a genome) is represented by aberrant gene expression, consisting of abnormal levels of expression for mature and/or precursor miRNA sequences compared with the corresponding normal tissues.
2. Germline and somatic mutations in miRNAs[21] or polymorphisms in the protein coding mRNAs targeted by miRNAs[22] may also contribute to cancer predisposition, initiation, and progression. In somatic cells, miRNA alterations could initiate or contribute to tumorigenesis, whereas germline mutations could represent cancer-predisposing events.
3. MiRNA profiling achieved by various methods has allowed the identification of signatures associated with diagnosis, staging, progression, prognosis, and response to treatment of human tumors (for review, see Reference 13). Therefore, miRNA "fingerprinting" represents a new addition to the diagnostic and prognostic tools to be used in medical oncology.

Other types of ncRNAs (such as ultraconserved genes [UCGs] and long intergenic noncoding RNAs [lincRNAs]) have also been linked to human cancers.[23] One of the most intriguing characteristics of miRNAs is the near-complete conservation of orthologous genes. For example, the active molecules of the *miR-16-1/miR-15a* cluster, shown to be an essential player in the initiation of CLL,[21] are completely conserved in humans, mice, and rats and highly conserved in 9 of 10 primate species sequenced. Comparative sequence analysis represents an essential tool in the identification of genomic DNA regions with important biologic functions. Several of these highly conserved genomic sequences were considered not genic (not producing a transcript) and were called *conserved nongenic sequences*.[24] A special subset of conserved sequences named *ultraconserved regions* (UCRs) include, by definition, intra- and intergenic sections of the human genome that are absolutely conserved (100% identical with no insertions or deletions) between orthologous regions of the human, rat, and mouse genomes.[25] Because of the high degree of conservation, the UCRs have been demonstrated to have fundamental functional importance for the ontogeny and phylogeny of mammals and other vertebrates. Recently, it was proved that most UCRs are transcribed and that hundreds of UCGs are consistently altered in a significant percentage of analyzed leukemias and carcinomas. UCGs are frequently located at fragile sites and genomic regions involved in cancers. It has also been proven that the inhibition of an overexpressed UCG induces apoptosis in colon cancer cells, and that the expression of some UCGs may be regulated by miRNAs abnormally expressed in CLL.[26] These new regulatory mechanisms support a model in which various types of noncoding genes are actively involved and cooperate with protein-coding genes in human tumorigenesis. Gathering all these notions together makes it clear that noncoding RNA genes, once seen as second-level genomic elements, are now at the center of attention in cancer research. Therefore, it is reasonable to attempt to expand the anticancer ammunition with RNA molecules capable of attenuating or completely abolishing the function of overexpressed ncRNAs—or, alternatively, to reexpress at physiologic levels the deleted or downregulated ncRNAs.

Main Types of Therapeutic RNA Molecules

Three different types of RNA molecules—the ribozymes, the siRNAs, and the anti-miRNA agents—have passed preclinical testing for efficiency in downregulating a target and now are entering clinical trials. At least two more types of RNA molecules recently added to the expanding list of anticancer ammunition—the miRNA-mimic agents and the 8-mer anti-miRNA LNA (locked nucleic acid) molecules—are going through preclinical tests. The antisense oligonucleotide (ASO) strategy was primarily developed using DNA molecules (for detailed discussion, see References 2 and 27). Although the history of RNAs as therapeutic

Table 55-2 Glossary of Terms in RNA-Inhibition Strategies

ASO: An antisense oligonucleotide is a single-stranded, chemically modified DNA-like molecule that is 17 to 22 nt in length and designed to be complementary to a selected messenger RNA and thereby specifically inhibit expression of that gene.
Messenger RNA (mRNA): The form of RNA that mediates the transfer of genetic information from the cell nucleus to ribosomes in the cytoplasm, where it serves as a template for protein synthesis. It is synthesized from a DNA template during the process of transcription.
Noncoding RNAs (ncRNAs): Any RNA molecule that is not translated into a protein.
Open reading frame (ORF): A section of a sequenced piece of DNA that begins with an initiation (methionine ATG) codon and ends with a nonsense codon. ORFs all have the potential to encode a protein or polypeptide; however, many may not actually do so.
Phase III clinical trials: Designed to study whether a new treatment is better than standard treatment by including hundreds of patients in the study and control groups.
Phase II clinical trials: Designed to determine whether a new treatment works by including tens of patients in the study or control groups.
Pol II: RNA polymerase II (also called RNAP II) catalyzes the transcription of DNA to synthesize precursors of mRNA and most small nuclear RNA.
Pol III: RNA polymerase III (also called RNAP III) transcribes DNA to synthesize 5S rRNA and other small RNAs. The genes transcribed by RNA Pol III fall in the category of "housekeeping" genes whose expression is required in all cell types and most environmental conditions.
Sense/antisense: Referring to the strand of a nucleic acid that directly specifies the product or referring to the strand of a double-stranded molecule that does not directly encode the product but is complementary to it.
Transcription: The process whereby RNA is synthesized from a DNA template.
Translation: The process of protein synthesis whereby the primary structure of the protein is determined by the nucleotide sequence in mRNA. The ribosome-mediated production of a polypeptide whose amino acid sequence is derived from the codon sequence of an mRNA molecule.
Untranslated region (UTR): The 5′ UTR is the portion of an mRNA from the 5′ end to the position of the first codon used in translation. The 3′ UTR is the portion of an mRNA from the 3′ end of the mRNA to the position of the last codon used in translation.
Watson-Crick pairing: The A-T and G-C pairing between the four types of DNA nucleotides.

molecules is two decades long, too few clinical trials have yet been conducted on large numbers of cancer patients to allow any convincing conclusion to be drawn. The first hints are encouraging and support the development of new and larger clinical trials. Most of the clinical and preclinical data were gathered from patients with viral infections, such as human immunodeficiency virus type-1 (HIV) or chronic hepatitis C virus (HCV). For a glossary of terms used in RNA inhibition strategies, see Table 55-2.

Ribozymes

In 1982, the first two ribozymes, the self-splicing intron of the *Tetrahymena* pre-rRNA and the RNaseP, were discovered by Cech's group[28] and Altman's group,[29] respectively. Both shared the Nobel prize in medicine in 1989 for this discovery. A naturally occurring or laboratory-prepared RNA enzyme, ribozyme is an RNA molecule that can catalyze a chemical reaction of substrate cleavage. The mechanism of action consists of three steps that are cyclically repeated. The first is represented by the Watson-Crick base pairing to a complementary target sequence, the second by the site-specific cleavage of the substrate, and the third by the release of the cleavage products. Although there are now seven naturally occurring classes of ribozymes,[30] the most commonly used class of ribozymes as therapeutic agents are the hammerhead (Hh) ribozymes. The Hhs are short-length RNAs, not more than 40 nt long, and are made of two substrate-binding arms plus a conserved catalytic domain of 24 bases.[31]

Angiozyme (RPI.4610; Sirna Therapeutics Inc, Boulder, Colo), an angiogenesis inhibitor, is an Hh ribozyme targeting a conserved region of human vascular endothelial growth factor receptor (VEGFR-1), selectively downregulating the VEGFR-1 by cleavage of its mRNA. It is the first synthetic ribozyme to be tested as a therapeutic agent in human cancer. Angiozyme was used in a Phase I clinical trial as a single agent in patients with biopsy-proven refractory solid tumor and showed promising results. The disease was stable in 25% of 28 eligible patients for a period of more than 6 months, with the longest treatment duration of more than 16 months, and showed no significant adverse reactions.[32] In another study, the same drug was combined with carboplatin and paclitaxel, indicating that this multidrug regimen can be administered in patients with advanced solid tumors with no substantial pharmacokinetic interactions.[33] The most common adverse effects were neutropenia, thrombocytopenia, pain, anemia, and fatigue. Angiozyme was well tolerated after intravenous (IV) infusion or a single subcutaneous (SC) bolus in healthy volunteers.[34] Combined with the data from the Phase I clinical trial, new studies will be designed and conducted to assess efficacy in various human cancers. Furthermore, as inhibition of either VEGFR-l or VEGFR-2

signaling can only partially block tumor angiogenesis and growth, simultaneous inhibition of both VEGFR-l and VEGFR-2 signaling could be highly effective in retarding the growth of some tumors.[35]

One obvious question is why, in spite of years of effort to decipher the structure and function of ribozymes, data accumulated in cancer patients are so scarce. One reason is the sequence specificity requirements of targeted RNAs that limit the amount of putative targets: for example, the Hh ribozymes work at their best on GUC and AUG triplets. Another reason is the limited accessibility of the drug to the mRNA complementary sequence due to the internal base pairing, producing secondary structures, and to the proteins that physically associate with the RNA. Furthermore, differences in targeted mRNA half-lives could affect the efficacy of the silencing, it being easier to eliminate a target with a shorter half-life than targets with a much longer half-life.

To improve the efficiency of ribozymes in cancer cells, a hybrid construct was produced, referred to as maxizyme.[36] This is a dimer of minimized ribozymes (minizymes) that can cleave two target sites located in two different mRNAs. The maxizyme also can allosterically cleave the target RNA only when it recognizes two target sites. For example, two distinct oncogenes, cyclin Dl (*CCND1*) and fibroblast growth factor 4 (*FGF4*, also named *HST-1*), which are overexpressed in breast cancer cells, were used as targets of a maxizyme. *CCND1* activity is required for cell cycle G_1/S transition, whereas *FGF4* is involved in tumor growth and invasion, and therefore blocking these genes could target a wide spectrum of pathways in malignant cells. When conventional ribozymes were used for suppression of expression of these genes, mRNAs in cancer cells and in normal cells were affected, whereas the trans-maxizyme cleaved these mRNAs only in the breast cancer cells. Whether such a drug can have similar results in clinical trials in patients with breast cancer is still an open question.

siRNAs and shRNAs

In 1998 Mello and Fire discovered RNA interference (RNAi) in vertebrates,[37] for which they received the Nobel prize in medicine in 2006. RNAi is a form of posttranscriptional gene silencing (PTG) in which double-stranded RNA (dsRNA), named *siRNA*, catalyzes the degradation of complementary mRNA targets. An *siRNA* is a dsRNA homologous to an mRNA of a target gene. In cytoplasm, the dsRNAs are processed by a complex consisting of Dicer and several other proteins into siRNAs, which are loaded into Argonaute 2 and RNA-induced silencing complex (RISC). The siRNA guide strand recognizes target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AG02. The processing of the siRNAs is similar to that of miRNAs, which is viewed as the "endogenous" process of RNA duplex maturation.[38,39]

A small-hairpin RNA (shRNA) represents an siRNA-like molecule expressed from a vector.[40] DNA cassettes encoding RNA polymerase III promoter-driven siRNA-like shRNAs allow long-term expression of therapeutic RNAs in targeted cells. One advantage over the ribozyme technology is the higher efficiency in targeting specific messengers. For example, when the efficiency of the shRNAs was compared with adenoviral delivery of an anti-MDR1 (multidrug resistance) ribozyme construct, the shRNA's downregulation of mRNA and protein expression was accompanied by a complete inhibition of the pump activity of MDR1 and a reversal of the multidrug-resistant phenotype. The ribozyme construct weakly affects gene expression, confirming that adenoviral delivery of shRNAs is much more effective than adenoviral delivery of ribozymes and that adenovirus-based vectors can be very powerful agents for the efficient delivery of therapeutic RNA molecules.[41]

Oncogenes expressed at abnormally high levels represent the main targets of siRNA-directed therapy. Results from preclinical studies are promising and show clear efficacy. For instance, in a mouse model of ovarian cancers overexpressing the tyrosine kinase receptor *EphA2* gene, the administration of liposomal-delivered siRNA targeting *EphA2* combined with paclitaxel determined a reduction of tumor size greater than 50% with intravenous or intraperitoneal routes of delivery. These data support the idea that chemotherapy and siRNA together can provide a powerful anticancer combination.[42] In another example, adenovirus-mediated siRNA against a *K-ras* mutated messenger (K-ras codon 12 GGT to GTT) markedly decreased *K-ras* gene expression and inhibited cellular proliferation of lung cancer cells that express the relevant mutation, but produced only minimal growth inhibition in cells that lack the specific abnormality.[43]

Although there are promising preclinical data, siRNA cancer therapy is overshadowed by few, but significant, concerning issues. The first involves the low bioavailability: in aqueous solution, siRNAs are extremely hydrophilic and heavily hydrated because of the exposure of the sugar-phosphate backbone to water, thus reducing their diffusion to the target tissue. Furthermore, because of degradation by serum nucleases, the in vivo half-lives of siRNAs are short. Chemical modifications of siRNAs to overcome these deficiencies include the conformationally "locked" nucleotide analog (LNA), which substantially increases the serum stability, without adversely affecting interactions with cellular silencing machinery. Introduction of a 2′-*O*-methyl group (2′-*O*-Me) and 2′-fluoro nucleotides enhances plasma stability and increases potency by several hundredfold in vivo

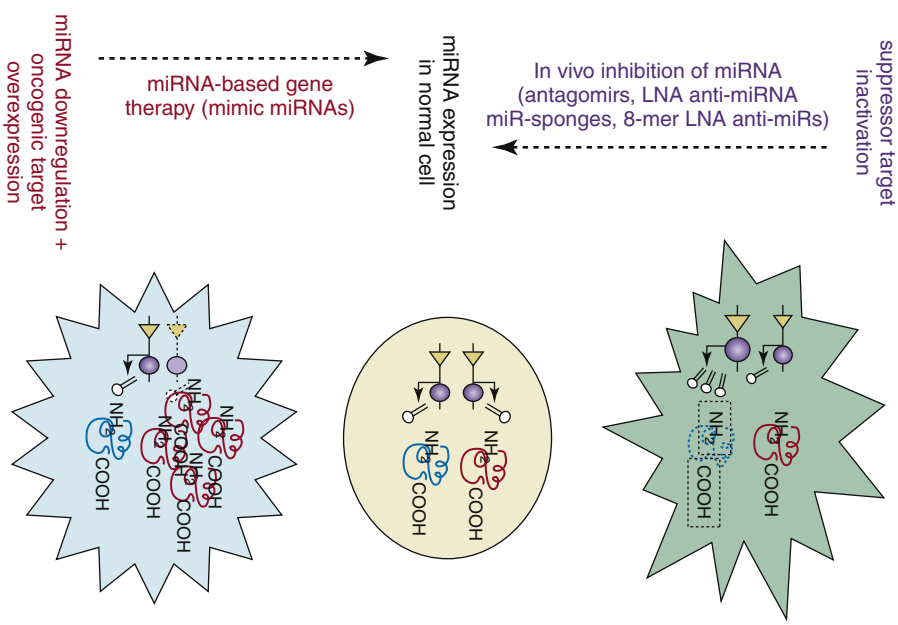


Figure 55-1 MicroRNAs (miRNAs) and anti-miRNAs as new therapeutic agents The type of therapeutic intervention is different with respect to the type of genetic alteration. If overexpressed, agents that specifically reduce miRNA expression to normal levels should be used, whereas if downregulated, agents that restore miRNA expression must be used. *(Modified from* Pharmacogenomics. *2007;53:521-537.)*

over the unmodified, "naked" siRNAs.[44] Another unsolved problem is represented by off-target effects (OTEs), meaning that, in addition to the complementary target, a specific siRNA can induce the silencing of several other imperfect complementary mRNAs that can be important for cell homeostasis. OTEs have been demonstrated by transcriptional profiling studies, and the nonspecific genes differentially expressed between treated and nontreated cells contain complementary regions to one of the two strands in the siRNA duplex. Chemical modification of nucleotides within this region of homology, by the introduction of a 2′-O-Me group, reduces the OTE without decreasing the silencing activity on messenger RNAs. Finally, a major concern is the stimulation of the innate immune system and the production of high amounts of interferon in response to siRNA duplexes. Sugar modifications (such as 2′-*O*-Me) and LNAs seem to reduce the immunostimulatory effects of siRNAs (for a comprehensive review of this topic, see Reference 44).

ASOs/AMOs Anti-miRNAs, LNAS Anti-miRNAs, and Antagomirs

A rationale for considering miRNAs as potential therapeutic targets is offered by the fact that miRNA overexpression in cancer cells has a pathogenic effect (see preceding sections). Therefore, several types of agents targeting miRNAs are now under development and ready to be tested for their in vivo effects and in clinical trials (Figure 55-1).[45,46]

Anti-miRNA oligonucleotides (AMOs) represent ASOs, single-stranded, chemically modified DNA-like molecules that are 17 to 22 nt in length and designed to be complementary to a selected miRNA. Thus they are able to specifically inhibit expression of that gene. Mechanistically, AMOs can be described as ASOs against miRNAs and therefore produce an ASO-miRNA duplex through Watson-Crick binding, leading to RNAse-H–mediated cleavage of the target miRNA gene. Important for potential clinical use, ASOs harboring a complete 2′-*O*-methoxyethyl and phosphorothioate modification have been demonstrated to silence in vivo *miR-122* in mouse liver.[47] The LNAs' anti-miRNAs represent modified antisense single-stranded oligonucleotides 17 to 22 nt in length, with a methylene bridge connecting the 2′ and 4′ carbons. *miR-21,* shown to be strongly overexpressed in glioblastomas, was silenced in vitro by using LNA-modified antisense oligonucleotides, leading to a significant reduction in cell viability and elevated intracellular levels of caspases.[48]

The antagomir represents RNA therapeutic molecules originally designed to inhibit miRNAs.[49,50] These are chemically modified and cholesterol-conjugated single-stranded 23-nt RNA molecules complementary to the targeted miRNAs. The modifications were introduced to increase the stability of the RNA and protect it from degradation. When intravenously administered to mice, antagomirs against *miR-122* (antagomir-122), a miRNA highly expressed in liver, induced a marked, specific, and persistent (up to 23 days) reduction of endogenous miRNA gene expression. The same was true for antagomir-16, targeting the ubiquitously expressed *miR-16*. Silencing of miRNAs by these new agents was followed by physiologic effects, such as a decrease in plasma cholesterol levels after antagomir-122 administration. The only tissue where antagomirs did not act when injected systemically was the brain—probably because of the difficulty

of crossing the blood-brain barrier—but they efficiently targeted miRNAs when injected locally into the mouse cortex. One clear advantage with respect to siRNA technology is that antagomirs did not induce an immune response.

A recent addition to this expanding category of therapeutic agents are the 8-mer LNA antimiRs, specifically designed to target the 5′ "seed" region of miRNAs. It was shown that an 8-mer LNA antimiR-155 oligonucleotide targeting miR-155 inhibits Waldenström macroglobulinemia (WM) and CLL cell proliferation in vitro, whereas systemically delivered antimiR-155 showed significantly decreased tumor growth in vivo.[51] Another approach is the use of "miRNA sponges" or "miRNA decoys"—transcripts expressed from strong promoters and containing multiple artificial miRNA binding sites that compete with the endogenous miRNA targets for miRNA binding.[52] Finally, "miRNA masks" are novel gene-specific anti-miRNAs that can selectively inhibit the interaction of the target miRNA with a specific mRNA. These consist of 2′-*O*-methyl-modified ASOs with locked 3′ and 5′ ends that effectively mask the specific mRNA from the endogenous miRNA and thus prevent its repression.[53]

miRNA-Mimic Agents

There are no reports in clinical practice of using the mimic miRNA agents that reproduce the effects of endogenous miRNAs and include all the chemical modifications necessary for stability and processing. However, the restoration of the expression of specific miRNAs abnormally expressed because of genomic deletions, hypermethylation, or other causes could be clinically used in the future. Several studies have validated the efficiency of miRNA mimics in in vitro and in vivo models. For example, intranasal administration of let-7 (which negatively regulates *RAS*) in a K-ras mutant mouse effectively inhibited tumor growth.[54] Similar studies have been performed for miR-34a in a prostate cancer model,[55] and for miR-26 in a liver cancer model.[56] Thus administration of miRNA-mimetic agents in patients might be a new avenue for clinical cancer management.

In Search of the Right Way and the Right Type of Delivery

Two practical issues regarding the use of RNAs as therapeutic molecules should be further dissected: the selection of the most adequate formula and the most efficient method of administration (local vs. systemic). Naked RNA delivery or vehicle delivered and viral vehicle versus nonviral delivery are the most important questions in the formula type debate. Initial therapeutic applications used 21-nt siRNA duplexes, with 2-nt 3′ overhangs, as well as longer siRNAs of 27 mers and shRNAs (29 nt). These drugs are able to induce only transient gene silencing, because their intracellular concentrations diminish with every cell division.[38] However, despite improvements in siRNA stability, naked siRNAs are of little therapeutic use and are likely to require some form of targeted delivery vehicle for sufficient tissue specificity and cellular uptake. Long-lasting and stable knockdown of target transcription is achieved by expression of shRNAs from various types of vectors, including viral vectors, with Pol II or Pol III promoters. Although therapeutically useful, the continuous expression can cause harmful side effects due to the saturation of the endogenous silencing pathway; thus abundant shRNA expression could be toxic. For example, the use of an adeno-associated virus (AAV) vector with a U6 promoter (Pol III type) induced high expression levels of shRNAs and caused death in about half of treated mice.[57] Different lentiviral vectors (LVs), adenoviral vectors (AVs), and AAVs are being tested. LVs efficiently integrate into the genome of nondividing cells, such as pancreatic islets or terminally differentiated cells. In contrast to LVs, AVs do not integrate into the host genome; they do, however, efficiently transduce dividing and nondividing cells. Different AAV serotypes can be potentially used for organ-directed miRNA expression. Another option is to use nonviral delivery vehicles for shRNAs. Liposomes, lipid complexes with small molecules, polymers, proteins, and antibodies were tested as potential delivery options and all have certain advantages (but also clear disadvantages[58]). With these delivery partners, the efficacy of shRNA is increased and doses reduced, but the cytotoxic effects are much greater (Table 55-3).

A second practical issue is the selection of local versus systemic administration of RNA drugs. Local administration has certain advantages, such as the need for lower doses with consequent lower adverse/toxic reactions and better bioavailability of the drug to the target tissue. Numerous publications have described the delivery of siRNA molecules directly to tumors in vivo, including direct applications of siRNA into to the peritoneal cavity, testes, or urethra (reviewed in Reference 59). In other instances, such as leukemias, local administration cannot be achieved and therefore systemic delivery is mandatory.

A Strategy for Using RNA as Therapeutic Molecules

RNA molecules are now at the center of molecular oncology, with applications for diagnosis and therapy beginning to

Table 55-3 Delivery Methods for RNA-Interference–Based Therapeutics

Method	Molecule Delivered	Advantage	Disadvantage
Nonviral Delivery			
Cholesterol	siRNA	Systemic delivery, stable	Nonselective delivery
SNALP	siRNA	Systemic delivery, highly stable	Nonselective delivery
Fab	siRNA	Selective delivery	Relatively complex formulation
Aptamer	siRNA	Selective delivery	Large-scale sequence screening required
Nanoparticle	siRNA	Receptor-specific, self-assembling	Sophisticated preparation required
Viral Delivery			
Lentivirus	RNA (shRNA produced)	Stable expression, transduces nondividing cells	Gene-disruption risk, localized delivery
Adenovirus	dsDNA (shRNA produced)	Episomal, no insertional mutagenesis	Immunogenic, dose-dependent hepatotoxicity, transient expression
AAV	ssDNA/dsDNA (shRNA produced)	Episomal, low genomic integration	Immunogenic, small sector capacity, transient expression

Modified with permission from Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Genet*. 2007;8:173-184.
dsDNA, Double-strand DNA; *SNALP*, stable nucleic acid-lipid particle; *ssDNA*, single-strand DNA.

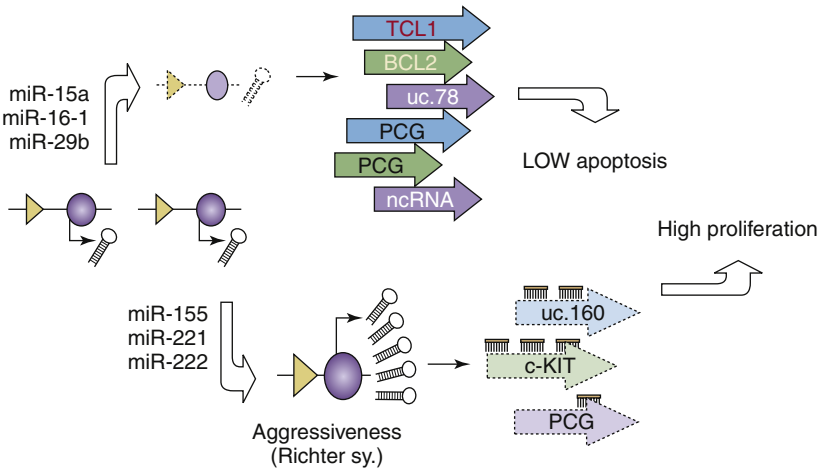


Figure 55-2 Chronic lymphocytic leukemia (CLL) as the best known example of interplay between noncoding RNAs (ncRNAs) and protein coding genes (PCGs) and the therapeutic implications During the initiation and progression of B-cell CLL alteration in at least three different types of genes, PCGs, microRNAs (miRNAs), and ultraconserved genes (UCGs) were identified. These are not independent, as complex regulatory interactions between miRNAs and PCGs and between miRNAs and UCGs occur. Furthermore, we postulate the existence of interactions between UCGs and PCGs, although these have not yet been demonstrated.

be proposed. Although exciting and full of promise, the field of RNA as a therapeutic molecule is not free of obstacles. Therefore, a good practical option is to attempt to improve strategies to optimize the efficiency of this new anticancer tool in the light of the unfulfilled promises of other types of new therapies. One approach to cancer therapy is to target various molecular defects in the multistep pathways of specific cancers—the *multiplex RNA inhibition targeting strategy*. Another strategy is to focus on a major molecular alteration clearly linked to disease pathogenesis by using multiple agents—the *Sandwich RNA inhibition strategy*. For example, to highlight, let us use the example of B-cell chronic lymphocytic leukemia (B-CLL), the most frequent adult leukemia in the Western world[60] and one of the best studied models of interplay between coding and ncRNAs in human cancers.[21] B-CLL is characterized by predominantly nondividing malignant B cells overexpressing the anti-apoptotic Bc12 protein. Deletion or downregulation of the *miR-15a/miR-16-1* cluster located at chromosome 13q14.3 represents an early event directly involved in the regulation of *BCL2* expression.[61] During the evolution of malignant clones, other miRNAs are deleted (such as *miR-29*) or overexpressed (such as *miR-155* or the cluster *miR-221/miR-222*), contributing to the aggressiveness of B-CLL (Richter syndrome). Such abnormalities influence the expression of other protein-coding genes: reportedly *miR-221* and *miR-222* directly down-modulate *c-KIT* receptor and *miR-29* regulates the levels of *TCL1* oncogene[62] or targets other ncRNAs such as UCGs *uc.160* and *uc.78* (Figure 55-2[26]). The consequences of this steady accumulation of abnormalities are represented

initially by low apoptosis and high survival of malignant B cells and later by the evolution of more aggressive clones with a higher proliferative capacity and survival by the overexpression of BCL2 and TCL1 oncogenes. Therefore, a mixed strategy of targeting both these oncogenes at the same time and using combined RNA drugs could be proposed. For example, designing a maxizyme anti-BCL2 and anti-TCL1 could be an option. Combining the systemic delivery of *miR-15* and *miR-16* family members targeting BCL2, together with *miR-29* and *miR-181* family members against TCL1, could increase the efficacy of therapeutic oncogenic downregulation. Furthermore, taking advantage of genome-wide profiling technologies, these therapies could be provided to only the subset of CLL patients having both types of lesions, specifically offered only to patients with *BCL2* overexpression and *mi-R15* and *miR-16* downregulations (the indolent form of CLL), or only to patients with *TCL1* overexpression and *miR-29* and *miR-181* downregulation (the aggressive form of CLL).

Finally, in light of the newly discovered interactions between various categories of ncRNAs, targeting not only miRNAs but also ultraconserved genes or lincRNAs regulated by miRNAs begins to be an alternative option. The competing endogenous RNA (ceRNA) hypothesis asserts that RNA transcripts can indirectly regulate each by competing for binding to miRNAs.[63] For example, synthetic RNA molecules that form hairpin structures simulating DNA transcription factor binding elements can be generated to target and regulate transcription factor activity, whereas synthetic lncRNAs that contain mutant miRNA binding sites can sequester and reduce expression levels of miRNAs.[64] Certainly, this is an exciting time for the RNA therapeutics, and we look forward to many more results of clinical studies investigating such strategies.

Acknowledgments

CM Croce is supported by Program Project Grants from the National Cancer Institute. GA Calin is the Alan M. Gewirtz Leukemia & Lymphoma Society Scholar. He is also supported as a Fellow at the University of Texas MD Anderson Research Trust, as a University of Texas System Regents Research Scholar, and by the NIH/NCI, the DoD, and the CLL Global Research Foundation. We thank Maitri Shah for critical reading of the manuscript. We apologize to the many colleagues whose work was not cited; because of space limitations we cited mainly the significant reviews and recent clinical studies.

Key References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2012;62:10-29.
2. Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer*. 2005;5:468-479.
3. O'Brien SM, Cunningham CC, Golenkov AK, et al. Phase I to II multicenter study of oblimersen sodium, a Bcl.2 antisense oligonucleotide, in patients with advanced chronic lymphocytic leukemia. *J Clin Oncol*. 2005;23:7697-7702.
4. O'Brien S, Moore JO, Boyd TE, et al. Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. *J Clin Oncol*. 2007;25:1114-1120.
5. Vansteenkiste J, Canon JL, Riska H, et al. Randomized phase II evaluation of aprinocarsen in combination with gemcitabine and cisplatin for patients with advanced/metastatic non small cell lung cancer. *Invest New Drugs*. 2005;23:263-269.
6. Ritch P, Rudin CM, Bitran JD, et al. Phase II study of PKC alpha antisense oligonucleotide aprinocarsen in combination with gemcitabine and carboplatin in patients with advanced non small cell lung cancer. *Lung Cancer*. 2006;52:173-180.
7. Paz-Ares L, Douillard JY, Koralewski P, et al. Phase III study of gemcitabine and cisplatin with or without aprinocarsen, a protein kinase C-alpha antisense oligonucleotide, in patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol*. 2006;24:1428-1434.
8. Marshall JL, Eisenberg SG, Johnson MD, et al. A phase II trial of ISIS 3521 in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2004;4:268-274.
9. Rao S, Watkins D, Cunningham D, et al. Phase II study of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C alpha, in patients with previously treated low grade non Hodgkin's lymphoma. *Ann Oncol*. 2004;15:1413-1418.
10. Villalona Calero MA, Ritch P, Figueroa JA, et al. A phase I/II study of LY900003, an antisense inhibitor of protein kinase C alpha, in combination with cisplatin and gemcitabine in patients with advanced non small cell lung cancer. *Clin Cancer Res*. 2004;10:6086-6093.
11. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. *Cell*. 2012;148:1172-1187.
12. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6:259-269.
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;6:857-866.
14. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*. 2003;113:673-676.
15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281-297.
16. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522-531.
17. Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell*. 2004;16:861-865.
18. Calin GA, Croce CM. MicroRNA cancer connection: the beginning of a new tale. *Cancer Res*. 2006;66:7390-7394.
19. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A*. 2004;101:9740-9744.
20. Hammond SM. microRNA detection comes of age. *Nat Methods*. 2006;3:12-13.
21. Calin GA, Ferracin M, Cimmino A, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353:1793-1801.
22. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102:19075-19080.

23. Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? *Oncogene*. 2012;31:4577-4587.
24. Dermitzakis ET, Reymond A, Antonarakis SE. Conserved non-genic sequences: an unexpected feature of mammalian genomes. *Nat Rev Genet*. 2005;6:151-157.
25. Bejerano G, Pheasant M, Makunin I, et al. Ultraconserved elements in the human genome. *Science*. 2004;304:1321-1325.
26. Calin GA, Cg Liu, Ferracin M, et al. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell*. 2007;12:215-229.
27. Opalinska JB, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov*. 2002;1:503-514.
28. Kruger K, Grabowski PJ, Zaug AJ, et al. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of. *Tetrahymena. Cell*. 1982;31:147-157.
30. Doudna JA, Lorsch JR. Ribozyme catalysis: not different, just worse. *Nat Struct Mol Biol*. 2005;12:395-402.
31. Tafech A, Bassett T, Sparanese D, et al. Destroying RNA as a therapeutic approach. *Curr Med Chem*. 2006;13:863-881.
32. Weng DE, Masci PA, Radka SF, et al. A phase I clinical trial of a ribozyme-based angiogenesis inhibitor targeting vascular endothelial growth factor receptor-1 for patients with refractory solid tumors. *Mol Cancer Ther*. 2006;4:948-955.
37. Fire A, Xu S, Montgomery MK, et al. Potent and specific genetic interference by double-stranded RNA in. *Caenorhabditis elegans. Nature*. 1998;391:806-811.
38. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Genet*. 2007;8:173-184.
40. Bernards R, Brummelkamp TR, Beijersbergen RL. shRNA libraries and their use in cancer genetics. *Nat Methods*. 2006;3:701-706.
41. Kaszubiak A, Holm PS, Lage H. Overcoming the classical multidrug resistance phenotype by adenoviral delivery of anti-MDR1 short hairpin RNAs and ribozymes. *Int J Oncol*. 2007;31:419-430.
42. Landen CN, Merritt WM, et al. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther*. 2006;5:1708-1713.
43. Zhang Z, Jiang G, Yang F, et al. Knockdown of mutant K-ras expression by adenovirus-mediated siRNA inhibits the in vitro and in vivo growth of lung cancer cells. *Cancer Biol Ther*. 2006;5:1481-1486.
44. Bumcrot D, Manoharan M, Koteliansky, et al. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol*. 2006;2:711-719.
45. Tili E, Michaille JJ, Gandhi V, et al. miRNAs and their potential for use against cancer and other diseases. *Future Oncol*. 2007;3:521-534.
46. Calin GA, Croce CM. Chromosomal rearrangements and microRNAs: a new cancer link with clinical implications. *J Clin Invest*. 2007;117:2059-2066.
47. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab*. 2006;3:87-98.
48. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*. 2005;65:6029-6033.
49. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with "antagomirs." *Nature*. 2005;438:685-689.
50. Krutzfeldt J, Kuwajima S, Braich R, et al. Specificity, duplex degradation and subcellular localization of antagomirs. *Nucleic Acids Res*. 2007;35:2885-2892.
51. Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-microRNA-155 silencing in low-grade B cell lymphomas. *Blood*. 2012;120:1678-1686.
52. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods*. 2007;4:721-726.
53. Xiao J, Yang B, Lin H, et al. Novel approaches for gene-specific interference via manipulating actions of microRNAs: examination on the pacemaker channel genes HCN2 and HCN4. *J Cell Physiol*. 2007;212:285-292.
54. Esquela-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle*. 2008;7:759-764.
55. Fujita Y, Kojima K, Hamada N, et al. Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells. *Biochem Biophys Res Commun*. 2008;377:114-119.
56. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. 2009;137:1005-1017.
57. Grimm D, Streetz KL, Jopling CL, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006;441:537-541.
58. Vorhies JS, Nemunaitis J. Nonviral delivery vehicles for use in short hairpin RNA-based cancer therapies. *Expert Rev Anticancer Ther*. 2007;7:373-382.
59. Aigner A. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. *J Biotechnol*. 2006;124:12-25.
60. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med*. 2005;352:804-815.
61. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*. 2005;102:13944-13949.
62. Pekarsky Y, Santanam U, Cimmino A, et al. Tcl1 expression in CLL is regulated by miR-29 and miR-181. *Cancer Res*. 2006;66:11590-11593.
63. Salmena L, Poliseno L, Tay Y, et al. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell*. 2011;146:353-358.
64. Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci Signal*. 2010;3:ra8.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics. *CA Cancer J Clin*. 2012;62:10-29.
2. Gleave ME, Monia BP. Antisense therapy for cancer. *Nat Rev Cancer*. 2005;5:468-479.
3. O'Brien SM, Cunningham CC, Golenkov AK, et al. Phase I to II multicenter study of oblimersen sodium, a Bcl.2 antisense oligonucleotide, in patients with advanced chronic lymphocytic leukemia. *J Clin Oncol*. 2005;23:7697-7702.
4. O'Brien S, Moore JO, Boyd TE, et al. Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. *J Clin Oncol*. 2007;25:1114-1120.
5. Vansteenkiste J, Canon JL, Riska H, et al. Randomized phase II evaluation of aprinocarsen in combination with gemcitabine and cisplatin for patients with advanced/metastatic non small cell lung cancer. *Invest New Drugs*. 2005;23:263-269.
6. Ritch P, Rudin CM, Bitran JD, et al. Phase II study of PKC alpha antisense oligonucleotide aprinocarsen in combination with gemcitabine and carboplatin in patients with advanced non small cell lung cancer. *Lung Cancer*. 2006;52:173-180.
7. Paz-Ares L, Douillard JY, Koralewski P, et al. Phase III study of gemcitabine and cisplatin with or without aprinocarsen, a protein kinase C-alpha antisense oligonucleotide, in patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol*. 2006;24:1428-1434.
8. Marshall JL, Eisenberg SG, Johnson MD, et al. A phase II trial of ISIS 3521 in patients with metastatic colorectal cancer. *Clin Colorectal Cancer*. 2004;4:268-274.
9. Rao S, Watkins D, Cunningham D, et al. Phase II study of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C alpha, in patients with previously treated low grade non Hodgkin's lymphoma. *Ann Oncol*. 2004;15:1413-1418.
10. Villalona Calero MA, Ritch P, Figueroa JA, et al. A phase I/II study of LY900003, an antisense inhibitor of protein kinase C alpha, in combination with cisplatin and gemcitabine in patients with advanced non small cell lung cancer. *Clin Cancer Res*. 2004;10:6086-6093.
11. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. *Cell*. 2012;148:1172-1187.
12. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6:259-269.
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;6:857-866.
14. Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell*. 2003;113:673-676.
15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281-297.
16. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5:522-531.
17. Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell*. 2004;16:861-865.
18. Calin GA, Croce CM. MicroRNA cancer connection: the beginning of a new tale. *Cancer Res*. 2006;66:7390-7394.
19. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci U S A*. 2004;101:9740-9744.
20. Hammond SM. microRNA detection comes of age. *Nat Methods*. 2006;3:12-13.
21. Calin GA, Ferracin M, Cimmino A, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353:1793-1801.
22. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102:19075-19080.
23. Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? *Oncogene*. 2012;31:4577-4587.
24. Dermitzakis ET, Reymond A, Antonarakis SE. Conserved non-genic sequences: an unexpected feature of mammalian genomes. *Nat Rev Genet*. 2005;6:151-157.
25. Bejerano G, Pheasant M, Makunin I, et al. Ultraconserved elements in the human genome. *Science*. 2004;304:1321-1325.
26. Calin GA, Cg Liu, Ferracin M, et al. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell*. 2007;12:215-229.
27. Opalinska JB, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov*. 2002;1:503-514.
28. Kruger K, Grabowski PJ, Zaug AJ, et al. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of. *Tetrahymena. Cell*. 1982;31:147-157.
29. Reed RE, Baer MF, Guerrier-Takada C, et al. Nucleotide sequence of the gene encoding the RNA subunit (M1 RNA) of ribonuclease P from *Escherichia coli*. *Cell*. 1982;30:627-636.
30. Doudna JA, Lorsch JR. Ribozyme catalysis: not different, just worse. *Nat Struct Mol Biol*. 2005;12:395-402.
31. Tafech A, Bassett T, Sparanese D, et al. Destroying RNA as a therapeutic approach. *Curr Med Chem*. 2006;13:863-881.
32. Weng DE, Masci PA, Radka SF, et al. A phase I clinical trial of a ribozyme-based angiogenesis inhibitor targeting vascular endothelial growth factor receptor-1 for patients with refractory solid tumors. *Mol Cancer Ther*. 2006;4:948-955.
33. Kobayashi H, Eckhardt SG, Lockridge JA, et al. Safety and pharmacokinetic study of RPI.4610 (ANGIOZYME), an anti-VEGFR-1 ribozyme, in combination with carboplatin and paclitaxel in patients with advanced solid tumors. *Cancer Chemother Pharmacol*. 2005;56:329-336.
34. Sandberg JA, Parker VP, Blanchard KS, et al. Pharmacokinetics and tolerability of an antiangiogenic ribozyme (ANGIOZYME) in healthy volunteers. *J Clin Pharmacol*. 2000;40(Pt 2):1462-1469.
35. Rafii S, Lyden D, Benezra R, et al. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer*. 2002;2:826-835.
36. Iyo M, Kawasaki H, Taira K. Construction of an allosteric trans-maxizyme targeting for two distinct oncogenes. *Nucleic Acids Res suppl*. 2002;20:115-116.
37. Fire A, Xu S, Montgomery MK, et al. Potent and specific genetic interference by double-stranded RNA in. *Caenorhabditis elegans. Nature*. 1998;391:806-811.
38. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Genet*. 2007;8:173-184.
39. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet*. 2002;3:737-747.
40. Bernards R, Brummelkamp TR, Beijersbergen RL. shRNA libraries and their use in cancer genetics. *Nat Methods*. 2006;3:701-706.
41. Kaszubiak A, Holm PS, Lage H. Overcoming the classical multidrug resistance phenotype by adenoviral delivery of anti-MDR1 short hairpin RNAs and ribozymes. *Int J Oncol*. 2007;31:419-430.
42. Landen CN, Merritt WM, et al. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther*. 2006;5:1708-1713.
43. Zhang Z, Jiang G, Yang F, et al. Knockdown of mutant K-ras expression by adenovirus-mediated siRNA inhibits the in vitro and in vivo growth of lung cancer cells. *Cancer Biol Ther*. 2006;5:1481-1486.
44. Bumcrot D, Manoharan M, Koteliansky, et al. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol*. 2006;2:711-719.
45. Tili E, Michaille JJ, Gandhi V, et al. miRNAs and their potential for use against cancer and other diseases. *Future Oncol*. 2007;3:521-534.
46. Calin GA, Croce CM. Chromosomal rearrangements and microRNAs: a new cancer link with clinical implications. *J Clin Invest*. 2007;117:2059-2066.
47. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab*. 2006;3:87-98.
48. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res*. 2005;65:6029-6033.
49. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with "antagomirs." *Nature*. 2005;438:685-689.
50. Krutzfeldt J, Kuwajima S, Braich R, et al. Specificity, duplex degradation and subcellular localization of antagomirs. *Nucleic Acids Res*. 2007;35:2885-2892.
51. Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-microRNA-155 silencing in low-grade B cell lymphomas. *Blood*. 2012;120:1678-1686.
52. Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods*. 2007;4:721-726.
53. Xiao J, Yang B, Lin H, et al. Novel approaches for gene-specific interference via manipulating actions of microRNAs: examination on the pacemaker channel genes HCN2 and HCN4. *J Cell Physiol*. 2007;212:285-292.

54. Esquela-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle*. 2008;7:759-764.
55. Fujita Y, Kojima K, Hamada N, et al. Effects of miR-34a on cell growth and chemoresistance in prostate cancer PC3 cells. *Biochem Biophys Res Commun*. 2008;377:114-119.
56. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. 2009;137:1005-1017.
57. Grimm D, Streetz KL, Jopling CL, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006;441:537-541.
58. Vorhies JS, Nemunaitis J. Nonviral delivery vehicles for use in short hairpin RNA-based cancer therapies. *Expert Rev Anticancer Ther*. 2007;7:373-382.
59. Aigner A. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. *J Biotechnol*. 2006;124:12-25.
60. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med*. 2005;352:804-815.
61. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*. 2005;102:13944-13949.
62. Pekarsky Y, Santanam U, Cimmino A, et al. Tcl1 expression in CLL is regulated by miR-29 and miR-181. *Cancer Res*. 2006;66:11590-11593.
63. Salmena L, Poliseno L, Tay Y, et al. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell*. 2011;146:353-358.
64. Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci Signal*. 2010;3:ra8.

Len Neckers and Mehdi Mollapour

56

Heat Shock Protein 90 and the Proteasome: Housekeeping Proteins That Are Also Molecular Targets for Cancer Therapy

Introduction

Cancer is a disease of genetic instability. Although only a few specific alterations seem to be required for generation of the malignant phenotype, at least in colon carcinoma there are approximately 10,000 estimated mutations at time of diagnosis.[1,2] The genetic plasticity of cancer cells allows them to frequently escape the precise molecular targeting of a single signaling node or pathway, making them ultimately nonresponsive to molecularly targeted therapeutics. Even Gleevec (Novartis Pharmaceuticals Corp.), a well-recognized, clinically active Bcr-Abl tyrosine kinase inhibitor, can eventually lose its effectiveness under intense, drug-dependent selective pressure, because of either mutation of the drug interaction site or expansion of a previously existing resistant clone.[3] Most solid tumors at the time of detection are already sufficiently genetically diverse to resist single-agent molecularly targeted therapy.[4] However, such an enhanced rate of mutation suggests that cancer cells should be highly dependent on efficient chaperoning and degradation of terminally misfolded proteins, because unchecked accumulation of misfolded proteins can initiate apoptosis.[5] Further, a multipronged attack on tumor cell signaling is likely to prove more efficacious than is targeting individual molecular pathways. Thus, pharmacologic agents that inhibit a cancer cell's ability to dispose of misfolded protein, allow for simultaneous attack of multiple signaling nodes, or possess both properties simultaneously should be of benefit. Hsp90 inhibitors and proteasome inhibitors display these characteristics and are actively being developed as novel anticancer agents.

Hsp90: a Chaperone of Cancer

A number of signaling proteins that are Hsp90 clients (see the website maintained by D. Picard, http://www.picard.ch/DP/downloads/Hsp90interactors.pdf, as well as several excellent reviews[6-8]) also mediate acquisition of the eight hallmarks of cancer[9] and promote cancer cell survival in the face of environmental stress (Figure 56-1). Therefore, it is not surprising that inhibition of Hsp90 may collapse or significantly weaken a cancer cell's "safety net." Several excellent reviews provide an in-depth description of the many signaling nodes regulated by Hsp90.[10-16]

Cancer cells are subjected to extreme environmental stress, such as hypoxia and acidosis, as well as the exogenously applied environmental stresses of chemotherapy or radiation. These stresses tend to generate free radicals that can cause significant physical damage to cellular proteins. Given the combined protective role of molecular chaperones toward damaged proteins and the dependence of multiple signal transduction pathways on Hsp90, it is therefore not surprising that molecular chaperones in general, and Hsp90 in particular, are highly expressed in most tumor cells. Hsp90 may provide a unique molecular target in cancer cells for an additional reason. In *Drosophila* and *Arabidopsis* model systems, Hsp90 has been suggested to be a buffer of genetic mutation.[17,18] Extrapolating this function to genetically unstable cancer cells, it is tempting to speculate that Hsp90 may be critical to a cancer's ability to survive in the presence of a constitutively high mutation rate.[19] Indeed, a recent study identified the Hsp90 inhibitor tanespimycin (17-AAG) in an unbiased in vitro screen for drugs active against aneuploid cancer cell lines.[20]

The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete *Streptomyces hygroscopicus* var. geldanus var. nova,[21] include geldanamycin (GA) and its semisynthetic derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) and the more water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, alvespimycin) and 18,21-didehydro-17-demethoxy-18,21-dideoxo-18,21-dihydroxy-17-(2-propenylamino) geldanamycin (IPI-504, retaspimycin) (Figure 56-2). These small molecules were the first identified inhibitors of Hsp90,[22,23] and they (and

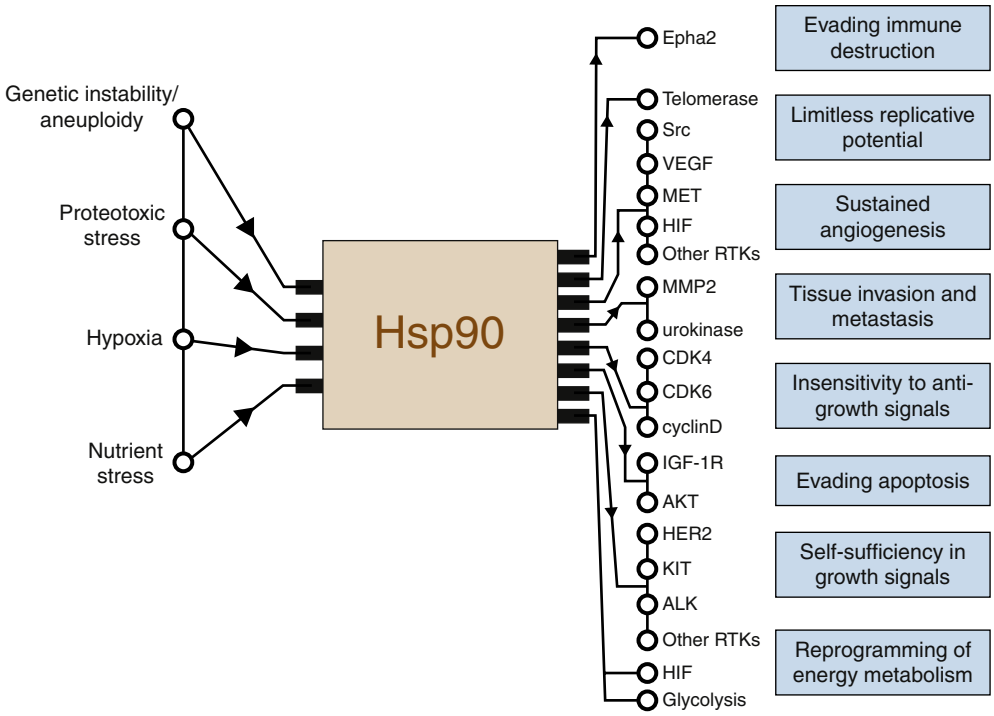


Figure 56-1 Hsp90 function is required for the establishment and maintenance of each of the eight hallmarks of cancer. Importantly, Hsp90 function is also critical for cancer cells to survive the genetic instability on which acquisition of the eight hallmarks depends, and the environmental stresses to which they are frequently subjected.

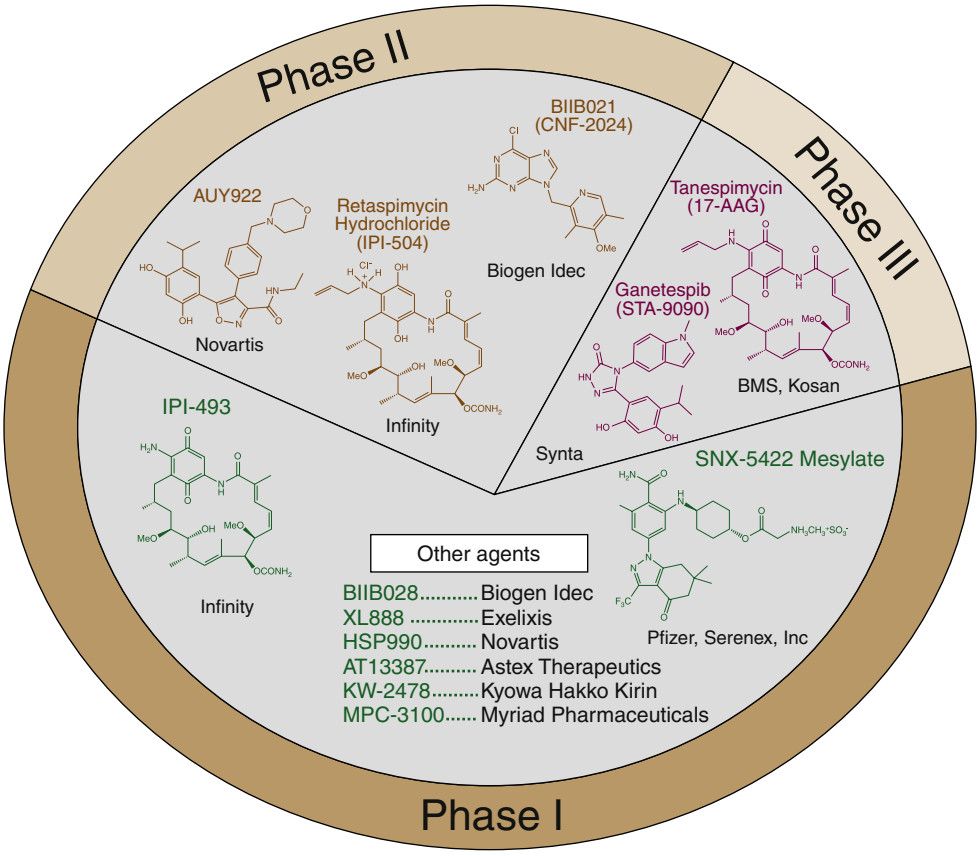


Figure 56-2 Hsp90 inhibitors in clinical development. See text for further details.

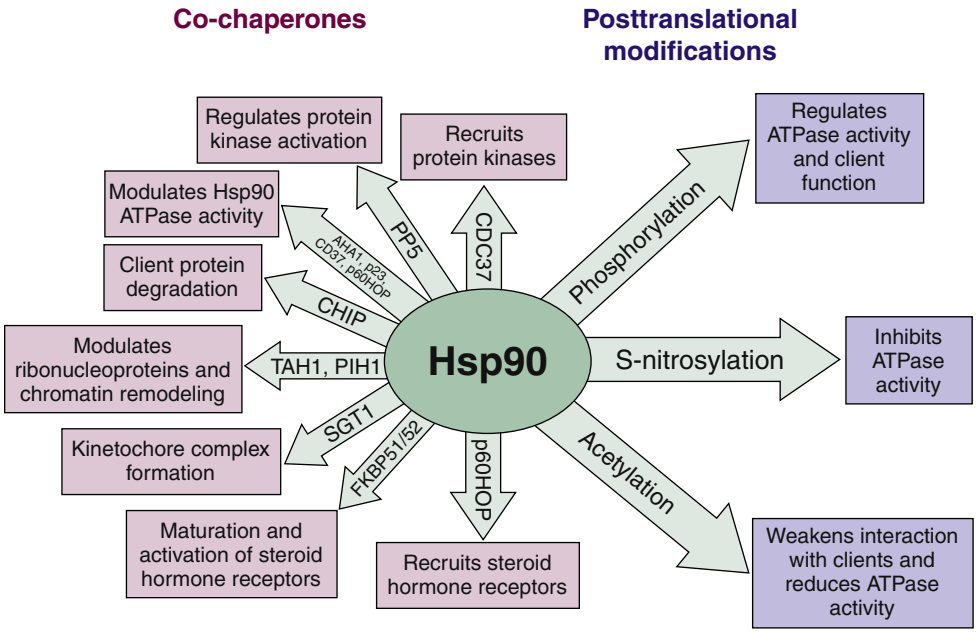


Figure 56-3 Co-chaperones and posttranslational modifications affect Hsp90 function. In eukaryotes, Hsp90 activity requires the contribution of numerous co-chaperones, each with a specific function. Acetylation, phosphorylation, and nitrosylation of specific residues on Hsp90 affect its interaction with client proteins and co-chaperones.

second-generation Hsp90 inhibitors) have shown activity in cancer clinical trials.[24,25] In particular, RECIST responses have been documented in patients with HER2$^+$ breast cancer previously treated with trastuzumab (Herceptin), and in EML4-ALK$^+$ non–small-cell lung cancer. Activity has also been seen in bortezomib-naïve and refractory multiple myeloma.

Several recent, excellently detailed reviews of the mechanics of Hsp90 function are in the scientific literature.[11,13,14,26-28] Hsp90 is a conformationally flexible ATPase that associates with a distinct set of co-chaperones depending on ATP binding to an amino-terminal purine binding pocket. Identification of this pocket as the GA binding site led Chiosis and colleagues to design a series of highly potent purine scaffold Hsp90 inhibitors with markedly improved drug-like properties.[29-32] Workman and colleagues used a high-throughput screen based on inhibition of Hsp90 ATPase activity to identify 3,4-diarylpyrazoles as a novel class of Hsp90 inhibitors.[33,34] Other groups have developed and validated a number of structurally distinct inhibitors of this purine pocket.[35] As of early 2012, more than 10 Hsp90 inhibitors had reached clinical trial (see Figure 56-2).

Hsp90 ATPase Activity and Chaperone Function

A model of Hsp90 function has emerged in which ATP binding to the amino-terminal pocket initiates a series of conformational changes that endow Hsp90 with ATPase activity. This process involves participation of a number of co-chaperones that interact with Hsp90 to form a "super-chaperone machine."[36] Certain co-chaperones play specific roles in this dynamic process (Figure 56-3). ATP hydrolysis completes the chaperone cycle, at which point the process (which is frequently iterative) can begin again. Many Hsp90 client proteins first associate with an Hsp70/Hsp40 chaperone complex.[37] This assemblage associates with Hsp90 via p60Hop, an Hsp90/Hsp70 interacting protein. At this point, when the client protein is being loaded on Hsp90, the chaperone is thought to be in an apo (nucleotide-free) conformation. ATP binding alters Hsp90 conformation, causing it to release p60Hop and the Hsp70/Hsp40 complex, and to recruit another set of co-chaperones, including p23 and certain immunophilins. In the case of kinase client proteins, the co-chaperone p50Cdc37 usually delivers the client to Hsp90. Although ATP hydrolysis is essential for chaperone activity, the ATPase activity of Hsp90 is very weak. Association of the co-chaperone Aha1 is necessary to increase Hsp90 ATPase activity sufficiently to drive the chaperone cycle forward.[38-41] In higher eukaryotes, the ordered association/dissociation of client proteins and co-chaperones is also affected by a series of sequential phosphorylation events.[42] Other posttranslational modifications also affect Hsp90 chaperone activity or client binding (see Figure 56-3).[43-45]

Hsp90 Inhibitors Target Client Proteins to the Proteasome

A highly orchestrated and tightly regulated process drives the ATP-dependent Hsp90 super-chaperone machine. Hsp90 inhibitors block the chaperone cycle by displacing ATP from the amino-terminal purine pocket, inducing client

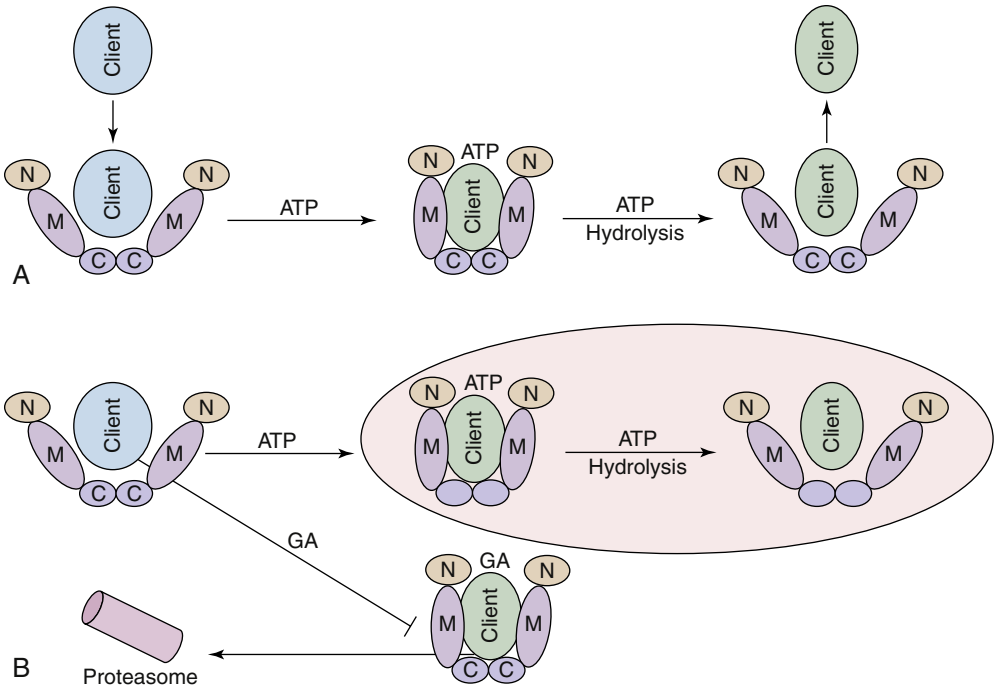


Figure 56-4 The Hsp90 chaperone machine **(A)** Client proteins associate weakly with an Hsp90 dimer in the absence of ATP. On ATP binding to an amino-terminal pocket in the chaperone, the N-lobes of each Hsp90 monomer transiently dimerize, resulting in tight binding of the client protein to Hsp90 and in acquisition of ATPase activity. On ATP hydrolysis, stimulated by various co-chaperones, the N-lobes of Hsp90 dissociate, releasing the now-folded client protein. **(B)** Geldanamycin (GA) and other N-terminal Hsp90 inhibitors block ATP binding to Hsp90, preventing dimerization and maintaining Hsp90 in a conformation that weakly associates with client protein. In the absence of ATP binding, the client protein dissociates from Hsp90, becomes polyubiquitinated by chaperone-dependent E3 enzymes, and is ultimately degraded by the proteasome.

protein ubiquitination and proteasome-mediated degradation (Figure 56-4). Although the mechanics of this process are not well understood, they appear to involve a "handing-off" of the client from inhibitor-bound Hsp90 to an Hsp70-ubiquitin ligase complex.[46] E3 ubiquitin ligases that have been implicated in mediating Hsp90 inhibitor-induced client protein ubiquitination in mammals include CHIP and Cullin 5.[47,48] Importantly, even if the proteasome is inhibited, client proteins are not rescued from Hsp90 inhibition, but instead accumulate in a misfolded, inactive state in detergent-insoluble subcellular complexes.[49] A selection of several oncogenic Hsp90 clients (in addition to HER2 and EML4-ALK, mentioned earlier) is shown in Table 56-1. The reader is directed to the references therein for more detailed information.

Hsp90 itself is also subject to ubiquitination and degradation. Wee1-mediated phosphorylation of a conserved tyrosine residue (Y38 in human Hsp90α) determines the ubiquitination and degradation of a nuclear pool of the chaperone, with consequences for Hsp90 function.[44,50]

Table 56-1 List of Selected Hsp90 Clients Involved in Cancer

Cancer	Hsp90 Client	References
Anaplastic large cell lymphomas	NMP-ALK	103, 104
Acute myeloid leukemia	FLT3	105, 106
Chronic myelogenous leukemia	BCR-ABL	107, 108
Human mastocytosis Gastrointestinal stromal tumors	KIT	109
Melanomas	B-RAF EGFR	89, 110-113
Prostate cancer	Androgen receptor	114-116
Clear cell renal cell carcinoma (ccRCC) Nonhereditary sporadic ccRCC	HIF1-α	117, 118
Glioblastoma	VEGF	119
Leukemia	BCL2 APAF	120
Small-cell lung cancer	MET	121-124
Multiple endocrine neoplasia type 2 (MEN2A, MEN2B) Familial medullary thyroid carcinoma (FMTC) Papillary carcinoma of thyroid	RET	125-128

Hsp90 Inhibitors May Prevent Oncogenic Switching

Recent studies have identified development of resistance to tyrosine kinase inhibition (TKI) as a significant roadblock to effective targeted therapy. One mechanism of resistance recently appreciated involves "oncogene switching," or the reactivation of signaling pathways by one or more redundant upstream activators. In breast cancer models, ErbB TKIs such as gefitinib have been shown to lose the ability to modulate ErbB-driven signaling pathways over time, even though ErbB inhibition is maintained.[51] Similarly, ErbB kinase activation has been reported to confer resistance to MET TKIs in MET oncogene-addicted gastric cancer cells.[52] This model of "oncogene plasticity" does not rely

on kinase mutation or on drastic changes in gene expression but merely on the inherent redundancy of biological systems, and it may explain some of the disappointing results seen in previous TKI clinical trials. Using several models, we and others have found that Hsp90 inhibition prevents oncogene switching and is itself a targeted therapy that is not prone to this phenomenon.[53-55] Although Hsp90 inhibitors may have single-agent activity in cancers that are strongly dependent on an Hsp90 client (e.g., HER2 or ALK), these drugs may find a broader role in combination with TKIs that target Hsp90 clients.

The Proteasome as an Anticancer Molecular Target

Regulated degradation of intracellular proteins is mediated by the proteasome, a 2.4-MDa molecular machine comprising approximately 60 subunits that together account for 2% of total cell protein. Not only do proteasomes regulate the half-lives of many signaling proteins in response to environmental stimuli, another primary function of the proteasome is to rapidly degrade hopelessly misfolded proteins that, if allowed to accumulate, can lead to apoptosis. Although the process leading from aggregation of misfolded proteins to cell death is not well understood and is likely to be multifactorial, one hypothesis is that accumulation of aggregated proteins results in the sequestering of numerous cellular chaperones and proteasome components in an insoluble and nonfunctional state, thus negatively affecting normal cell homeostasis and promoting cellular apoptosis. Deregulated protein aggregation has been shown to cause mitochondrial membrane depolarization, release of cytochrome c, and activation of caspase cascades. Indeed, deficiency in proteasome processing of misfolded proteins underlies a number of neurodegenerative diseases characterized by abnormal deposition of insoluble misfolded proteins that cause the apoptotic death of neuronal cells. The frequent acidosis and hypoxia to which cancer cells are subjected cause free radical–mediated damage to cellular proteins. If this damage cannot be repaired, such proteins are cleared from the cell via degradation in the proteasome. Thus, in order to maintain homeostasis, cancer cells are highly dependent on the ability of the proteasome machinery to operate with maximal efficiency.

The proteasome is composed of a 20S core particle containing three proteolytic activities that recognize hydrophobic, basic, and acidic amino acids, respectively. The 26S proteasome is composed of a 20S core particle capped on either end by a 19S ubiquitin chain recognition particle that also uses ATP to unwind substrate protein, allowing it to enter the 20S core where it is degraded into small peptides 2 to 25 residues in length. On exiting the proteasome, these small peptides are rapidly degraded to their amino acid components by cytosolic peptidases. The polyubiquitin chain is also removed and disassembled to monoubiquitin for reuse. The interested reader is referred to several excellent reviews on proteasome function and on validation of the proteasome and ubiquitination machinery as drug targets.[56-58]

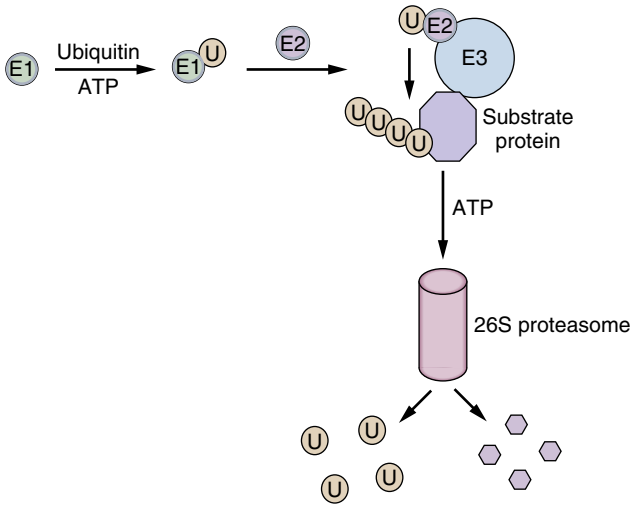


Figure 56-5 The ubiquitin-proteasome pathway Using ATP, a series of enzymes (E1, E2, and E3) attach multiple units of the small protein ubiquitin to a substrate protein destined for degradation. Substrate specificity is provided by the E3 complex. Once polyubiquitinated, the substrate protein is recognized by the 19S cap of the 26S proteasome and in an ATP-dependent process is unwound and fed into the proteasome for degradation. The 19S core of the proteasome contains chymotryptic, tryptic, and caspase-like proteolytic activities, thus ensuring efficient degradation of the substrate protein into small peptides that, on exiting from the proteasome, are cleaved to their constituent amino acids by cytosolic peptidases. Polyubiquitin chains are disassembled to resupply the cellular monoubiquitin pool. Proteasome inhibitors inhibit one or more enzymatic activities of the 19S core (see text).

As stated earlier, the 26S proteasome recognizes polyubiquitin chains, and most proteins destined for proteasomal degradation are first tagged by sequential covalent addition of four or more ubiquitin moieties (a 76-amino-acid, highly conserved protein present in the cytoplasm and nucleus of all eukaryotes). This process involves ATP-dependent charging of a ubiquitin-activating enzyme (E1), which then transfers ubiquitin to a ubiquitin-conjugating enzyme (E2), which in the presence of a ubiquitin-ligating enzyme (E3) transfers a single ubiquitin moiety to the protein to be degraded (Figure 56-5). Interestingly, there is only one E1 enzyme in mammalian cells, approximately 50 E2 enzymes, but perhaps 1000 E3 enzymes. Thus, substrate specificity is primarily regulated by the selectivity of the E3 enzyme. Interaction of the E3 ubiquitin ligase with its substrate is frequently dependent on posttranslational modification (e.g., phosphorylation) of the substrate protein or, in the case of misfolded proteins, on initial interaction of the substrate protein with one or more molecular chaperones, including Hsp90.

Anticancer Activity of the Proteasome Inhibitor Bortezomib

Bortezomib, a dipeptidyl boronic acid–based reversible inhibitor, was the first proteasome inhibitor to enter clinical trials in hematologic malignancies. It has shown significant activity toward multiple myeloma, and in 2005 the U.S. Food and Drug Administration (FDA) approved its use in patients with relapsed multiple myeloma. Multiple additional clinical trials are currently under way examining the efficacy of this agent in various hematologic and solid tumors.[59] Preliminary data suggest promising activity in mantle-cell lymphoma and follicular lymphoma. Several clinical trials are also testing bortezomib in combination with other therapeutic agents, including dexamethasone, doxorubicin, melphalan, and Hsp90 inhibitors. For an in-depth review of bortezomib's possible mechanism(s) of action and clinical evaluation, the reader is referred to an excellent review by Roccaro and colleagues.[59] A comprehensive review of the current status (as of 2012) of proteasome inhibitors in multiple myeloma is also recommended.[60]

Second-Generation Proteasome Inhibitors

In recent years, several second-generation proteasome inhibitors have been developed and are being evaluated in the clinic. MLN9708, like bortezomib, is a boronate derivative, but it has demonstrated greater tissue penetration in preclinical studies and is the first orally available proteasome inhibitor to be evaluated in multiple myeloma.[61] Preliminary clinical data reveal encouraging activity, and durable responses have been seen in heavily pretreated patients.[62] CEP-18770 is a third boronate derivative, also orally active, that is in development.[63]

Carfilzomib, an epoxyketone, is an irreversible proteasome inhibitor that is structurally and mechanistically distinct from the boronate-based drugs. Carfilzomib has demonstrated reduced off-target activity compared to bortezomib, and, importantly, activity has been seen against bortezomib-resistant cell lines and primary multiple myeloma cells.[64,65] Carfilzomib has demonstrated durable antitumor activity in patients with relapsed/refractory multiple myeloma and, unlike bortezomib, has caused limited neurotoxicity.[66] An analog of carfilzomib, ONX-0912, is also under development.[67]

Marizomib (NPI-0052), a natural product lactone isolated from a marine bacterium, unlike the boronate compounds and carfilzomib, is an irreversible proteasome inhibitor that abrogates both the chymotrypsin-like and trypsin-like protease activities while only minimally affecting the caspase-like activity of the proteasome. As a result, it does not exhibit cross resistance with other proteasome inhibitors, has a unique safety profile, and has demonstrated antitumor activity in preclinical models of multiple myeloma, other hematologic diseases, and solid tumors.[68-70] Marizomib also demonstrates oral bioavailability.

Combined Inhibition of Hsp90 and the Proteasome

Proteasome-mediated degradation is the common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors.[71,72] Thus, at first glance, combining a proteasome inhibitor with an Hsp90 inhibitor may seem counterintuitive. However, proteasome inhibition does not protect Hsp90 clients in the context of chaperone inhibition—instead, client proteins become insoluble.[49,73] Because the deposition of insoluble proteins is toxic to cells,[74,75] interest has arisen in combining proteasome and Hsp90 inhibitors, the goal being to promote enhanced accumulation of insoluble proteins and trigger apoptosis. This hypothesis is particularly appealing given the clinical efficacy of proteasome inhibitors alone.[59] Initial experimental support for such a hypothesis was provided by Mitsiades and colleagues,[76] who reported that Hsp90 inhibitors enhanced multiple myeloma cell sensitivity to proteasome inhibition. Clinically, a combination of tanespimycin and bortezomib has been associated with durable responses in heavily pretreated patients with multiple myeloma, including those with bortezomib-refractory disease.[77-80] Additional Hsp90 inhibitors are being evaluated in this setting.[81-83]

Importantly, transformed cells are more sensitive to the cytotoxic effects of this drug combination than are nontransformed cells. Thus, 3T3 fibroblasts are fully resistant to the combined administration of 17-AAG and bortezomib at concentrations that prove cytotoxic to 3T3 cells transformed by *HPV16* virus encoding viral proteins E6 and E7.[84] In the same study, Mimnaugh and co-workers demonstrated that the endoplasmic reticulum is one of the main targets of this drug combination. In the presence of combined doses of both agents that show synergistic cytotoxicity, these investigators noted a nearly complete disruption of the architecture of the endoplasmic reticulum. Because all secreted and transmembrane proteins must pass through this organelle on their route to the extracellular space, it is not surprising that a highly secretory cancer such as multiple myeloma would be particularly sensitive to combined inhibition of Hsp90 and the proteasome. One might speculate that other highly secretory cancers, including hepatocellular carcinoma and pancreatic carcinoma, would also respond favorably to this drug combination.

Additional Rationales for Inhibiting the Ubiquitin-Proteasome System in Cancer

As stated earlier, the proteasome inhibitor bortezomib has demonstrated single-agent activity in multiple myeloma and in other hematologic malignancies. Although general interference in the clearance of misfolded proteins is likely to be a major contributor to the efficacy of this agent, other more specific effects of proteasome inhibition should also be considered.[59] In these hematologic cells, the transcription factor NFκB plays a particularly important role. Not only does it inhibit apoptosis, but it actively upregulates transcription of growth factors such as interleukin 6 and angiogenic factors such as VEGF. As a transcription factor, the activity of NFκB requires nuclear entry. This in turn is regulated by targeted, proteasome-mediated degradation of IκB, a protein that interacts with NFκB and restricts it to the cytosol. Treatment of cells with bortezomib has been shown to prevent the degradation of IκB, thus resulting in retention of NFκB in the cytosol.

Other important tumor suppressor proteins degraded by the proteasome include p53 and p27. Thus, proteasome inhibition promotes the accumulation of these proteins. Recently, investigators have identified more specific approaches to prevent inappropriate p53 and p27 degradation by searching for inhibitors of the E3 ligases that recognize these proteins. The E3 interacting with p53 is termed *MDM2* (double minute protein 2). Two small molecules that interfere with MDM2/p53 interaction have recently been identified.[85,86] Although mechanistically distinct, these agents both result in accumulation of wild-type p53 in tumor cells in vitro and shrink tumors growing in mice. The tumor suppressor p27 is degraded by the E3 ligase SKP2 (S phase kinase-associated protein 2). SKP2, which also targets other antiproliferative molecules in the cell—including the retinoblastoma family protein p130, the cyclin-dependent kinase inhibitors p21 and p57, and the inhibitory transcription factor FoxO (forkhead box protein O)—is overexpressed in many human cancers.[57] Molecular knockdown of SKP2 using RNA interference techniques or intracellular injection of SKP2-specific antibodies slows the proliferation of cancer cells in vitro.[57,87] Thus, SKP2 is a valid therapeutic target in its own right, and drug discovery efforts specifically targeting this ubiquitin ligase are under way.[88]

Why Are Tumor Cells Uniquely Sensitive to Hsp90 and Proteasome Inhibition?

It is apparent, from both preclinical and clinical observations, that Hsp90 inhibitors can be administered in vivo at doses and schedules that significantly affect tumor growth but display acceptable target-related toxicity in normal tissues or in the whole organism. This is the case for several small-molecule inhibitors, including 17-AAG and 17-DMAG, the synthetic purine mimetic PU24FCl, and is even true for a novel peptidomimetic inhibitor of the N-terminal Hsp90 nucleotide binding site, shepherdin.[89-93] When murine model systems are examined in vivo, Hsp90 inhibitors are found to concentrate in tumor tissue, while being rapidly cleared from normal tissue with a half-life similar to that of drug in plasma.[89,90,92,93] The Hsp90 inhibitor 17-AAG also has been reported to actively concentrate in tumor cells in vitro.[94]

Because preferential accumulation of Hsp90 inhibitors in tumor versus normal tissue may provide the observed therapeutic (or at least biologic) index, it is important to understand the reason for this phenomenon. A possible explanation put forth by Kamal and colleagues suggests that enhanced drug binding to tumor cell Hsp90 reflects the activity state of the Hsp90 chaperone machine in tumor versus normal cells.[95] They proposed that the enhanced ATPase activity of the chaperone in tumor cells, which is dependent on preferential recruitment of Hsp90 to a multicomponent chaperone complex, is responsible for the increased affinity of Hsp90 inhibitors in tumor cells. More recently, the suggestion that tumor cell Hsp90 more avidly binds Hsp90 inhibitors compared to the Hsp90 in nontransformed cells has received experimental support,[96] and posttranslational modifications of Hsp90 that affect drug binding have been described.[43,44]

The expression of NAD(P)H:Quinone Oxidoreductase I (NQO1), also known as DT-diaphorase, dramatically enhances cellular sensitivity to 17-AAG.[89,97] NQO1 generates the hydroquinone form of 17-AAG, which has been shown to bind more avidly to Hsp90 when compared to 17-AAG itself.[98] Further, the presence of NQO1 in a cell seems also to lead to increased total accumulation of intracellular ansamycin molecules, presumably reflecting the increased Hsp90 affinity of 17-AAG hydroquinone. The presence of NQO1 in tumor cells dramatically affects cellular sensitivity to 17-AAG.[89,97,98] Because high levels of NQO1 have been observed in diverse tumor types (e.g., liver, lung, colon, breast) compared to normal tissues of the same origin,[99] these data suggest an explanation for the disparate sensitivity of tumor and normal tissue to 17-AAG and to retaspimycin (IPI-504), a stabilized hydroquinone form of 17-AAG that is in clinical trial.[100,101] A similar preference of non-ansamycin Hsp90 inhibitors for tumor cell Hsp90 supports the hypothesis that unique modifications of Hsp90 in tumor versus normal cells also contribute to this phenomenon.

Proteasome inhibitors also display selective cytotoxicity toward tumor cells, both in vitro and in vivo. Why are they not more toxic to normal cells? Kisselev and colleagues[102]

have recently shown that, at therapeutic doses of bortezomib in vivo, and following the intermittent schedule of administration approved for patients, only the chymotryptic activity of the proteasome is significantly inhibited and the overall rate of protein degradation is reduced by less than 40%. Because cancer cells may require their proteasomes to be at full capacity in order to handle the load of continually generated misfolded proteins, moderate reduction in proteasome activity, even for a brief period, may prove deleterious. In contrast, nontransformed cells may be able to tolerate reduced proteasome function for an extended period of time. Some of our data described earlier in this review support this hypothesis. In the preclinical studies in which we observed dramatically enhanced toxicity (and dramatically enhanced insoluble ubiquitinated protein deposition) by combining low-dose Hsp90 inhibitors with bortezomib, we found that maximal benefit of the combination required only a 50% reduction in proteasome activity.[84] Importantly, the satisfactory safety profiles of second-generation irreversible inhibitors—including marizomib, which inhibits two of three proteasome-associated protease activities—suggest that nontransformed cells may be able to tolerate prolonged, significantly reduced proteasome activity, further amplifying the therapeutic index of these drugs.

Conclusion

The proteasome and Hsp90 together comprise approximately 4% of total cellular protein. Separately and together they regulate critical mechanisms responsible for maintaining cellular homeostasis in the face of severe environmental stress and constitutive genetic instability. Although Hsp90 and the proteasome certainly can be labeled as "housekeeping" proteins, both proteins have proven to be exciting and clinically relevant anticancer molecular targets. Because it is now clear that cancer cells, by their very nature, are more dependent on optimal function of homeostatic mechanisms compared to normal cells, further exploitation of these pathways as drug targets will likely provide additional therapeutic strategies to attack this disease.

Key References

4. Kitano H. Cancer robustness: tumour tactics. *Nature*. 2003;426:125.
5. Hetz C, Soto C. Protein misfolding and disease: the case of prion disorders. *Cell Mol Life Sci*. 2003;60:133-143.
6. Nardai G, Vegh EM, Prohaszka Z, Csermely P. Chaperone-related immune dysfunction: an emergent property of distorted chaperone networks. *Trends Immunol*. 2006;27:74-79.
7. Zhao R, Davey M, Hsu YC, et al. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell*. 2005;120:715-727.
8. Sreedhar AS, Soti C, Csermely P. Inhibition of Hsp90: a new strategy for inhibiting protein kinases. *Biochim Biophys Acta*. 2004;1697:233-242.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
10. Isaacs JS. Heat-shock protein 90 inhibitors in antineoplastic therapy: is it all wrapped up? *Expert Opin Investig Drugs*. 2005;14:569-589.
11. Chiosis G, Vilenchik M, Kim J, Solit D. Hsp90: the vulnerable chaperone. *Drug Discov Today*. 2004;9:881-888.
12. Workman P. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. *Cancer Lett*. 2004;206:149-157.
13. Bagatell R, Whitesell L. Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol Cancer Ther*. 2004;3:1021-1030.
14. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med*. 2004;82:488-499.
15. Goetz MP, Toft DO, Ames MM, Erlichman C. The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann Oncol*. 2003;14:1169-1176.
16. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell*. 2003;3:213-217.
19. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer*. 2005;5:761-772.
20. Tang YC, Williams BR, Siegel JJ, Amon A. Identification of aneuploidy-selective antiproliferation compounds. *Cell*. 2011;144:499-512.
23. Porter JR, Ge J, Lee J, et al. Ansamycin inhibitors of Hsp90: nature's prototype for anti-chaperone therapy. *Curr Top Med Chem*. 2009;9:1386-1418.
24. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer*. 2010;10:537-549.
25. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? *Clin Cancer Res*. 2012;18:64-76.
26. Siligardi G, Hu B, Panaretou B, et al. Co-chaperone regulation of conformational switching in the Hsp90 ATPase cycle. *J Biol Chem*. 2004;279:51989-51998.
27. Wegele H, Muller L, Buchner J. Hsp70 and Hsp90—a relay team for protein folding. *Rev Physiol Biochem Pharmacol*. 2004;151:1-44.
28. Prodromou C, Pearl LH. Structure and functional relationships of Hsp90. *Curr Cancer Drug Targets*. 2003;3:301-323.
29. He H, Zatorska D, Kim J, et al. Identification of potent water soluble purine-scaffold inhibitors of the heat shock protein 90. *J Med Chem*. 2006;49:381-390.
30. Llauger L, He H, Kim J, et al. Evaluation of 8-arylsulfanyl, 8-arylsulfoxyl, and 8-arylsulfonyl adenine derivatives as inhibitors of the heat shock protein 90. *J Med Chem*. 2005;48:2892-2905.
31. Chiosis G, Lucas B, Huezo H, et al. Development of purine-scaffold small molecule inhibitors of Hsp90. *Curr Cancer Drug Targets*. 2003;3:371-376.
33. Cheung KM, Matthews TP, James K, et al. The identification, synthesis, protein crystal structure and in vitro biochemical evaluation of a new 3,4-diarylpyrazole class of Hsp90 inhibitors. *Bioorg Med Chem Lett*. 2005;15:3338-3343.
34. Dymock BW, Barril X, Brough PA, et al. Novel, potent small-molecule inhibitors of the molecular chaperone Hsp90 discovered through structure-based design. *J Med Chem*. 2005;48:4212-4215.
35. Kim YS, Alarcon SV, Lee S, et al. Update on Hsp90 inhibitors in clinical trial. *Curr Top Med Chem*. 2009;9:1479-1492.
37. Hernandez MP, Chadli A, Toft DO. HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. *J Biol Chem*. 2002;277:11873-11881.
42. Xu W, Mollapour M, Prodromou C, et al. Dynamic tyrosine phosphorylation modulates cycling of the HSP90-P50(CDC37)-AHA1 chaperone machine. *Mol Cell*. 2012;47:434-443.
43. Mollapour M, Neckers L. Post-translational modifications of Hsp90 and their contributions to chaperone regulation. *Biochim Biophys Acta*. 2012;1823:648-655.

44. Mollapour M, Tsutsumi S, Donnelly AC, et al. Swe1Wee1-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function. *Mol Cell*. 2010;37:333-343.
45. Mollapour M, Tsutsumi S, Kim YS, et al. Casein kinase 2 phosphorylation of Hsp90 threonine 22 modulates chaperone function and drug sensitivity. *Oncotarget*. 2011;2:407-417.
47. Xu W, Marcu M, Yuan X, et al. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A*. 2002;99:12847-12852.
48. Ehrlich ES, Wang T, Luo K, et al. Regulation of Hsp90 client proteins by a Cullin5-RING E3 ubiquitin ligase. *Proc Natl Acad Sci U S A*. 2009;106:20330-20335.
49. An WG, Schulte TW, Neckers LM. The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome. *Cell Growth Differ*. 2000;11:355-360.
50. Mollapour M, Tsutsumi S, Neckers L. Hsp90 phosphorylation, Wee1 and the cell cycle. *Cell Cycle*. 2010;9:2310-2316.
51. Stommel JM, Kimmelman AC, Ying H, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science*. 2007;318:287-290.
52. Bachleitner-Hofmann T, Sun MY, Chen CT, et al. HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells. *Mol Cancer Ther*. 2008;7:3499-3508.
53. Pashtan I, Tsutsumi S, Wang S, et al. Targeting Hsp90 prevents escape of breast cancer cells from tyrosine kinase inhibition. *Cell Cycle*. 2008;7:2936-2941.
54. Wang S, Pashtan I, Tsutsumi S, et al. Cancer cells harboring MET gene amplification activate alternative signaling pathways to escape MET inhibition but remain sensitive to Hsp90 inhibitors. *Cell Cycle*. 2009;8:2050-2056.
55. Koizumi H, Yamada T, Takeuchi S, et al. Hsp90 inhibition overcomes HGF-triggering resistance to EGFR-TKIs in EGFR-mutant lung cancer by decreasing client protein expression and angiogenesis. *J Thorac Oncol*. 2012;7:1078-1085.
56. Goldberg AL. Functions of the proteasome: From protein degradation and immune surveillance to cancer therapy. *Biochem Soc Trans*. 2007;35:12-17.
57. Nalepa G, Rolfe M, Harper JW. Drug discovery in the ubiquitin-proteasome system. *Nat Rev Drug Discov*. 2006;5:596-613.
58. Reinstein E, Ciechanover A. Protein degradation and human diseases: the ubiquitin connection. *Ann Intern Med*. 2006;145:676-207.
59. Roccaro AM, Hideshima T, Richardson PG, et al. Bortezomib as an antitumor agent. *Curr Pharm Biotech*. 2006;7:441-448.
60. Moreau P, Richardson PG, Cavo M, et al. Proteasome inhibitors in multiple myeloma: ten years later. *Blood*. 2012;120:947-959.
61. Kupperman E, Lee EC, Cao Y, et al. Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res*. 2010;70:1970-1980.
62. Richardson PG, Baz R, Wang L, et al. Investigational agent MLN9708, an oral proteasome inhibitor, in patients (pts) with relapsed and/or refractory multiple myeloma (MM): results from the expansion cohorts of a phase 1 dose-escalation study. *ASH Annual Meeting Abstracts*. 2011;118:301.
63. Piva R, Ruggeri B, Williams M, et al. CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. *Blood*. 2008;111:2765-2775.
64. Demo SD, Kirk CJ, Aujay MA, et al. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res*. 2007;67:6383-6391.
65. Kuhn DJ, Chen Q, Voorhees PM, et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood*. 2007;110:3281-3290.
66. Jain S, Diefenbach C, Zain J, O'Connor OA. Emerging role of carfilzomib in treatment of relapsed and refractory lymphoid neoplasms and multiple myeloma. *Core Evid*. 2011;6:43-57.
67. Chauhan D, Singh AV, Aujay M, et al. A novel orally active proteasome inhibitor ONX 0912 triggers in vitro and in vivo cytotoxicity in multiple myeloma. *Blood*. 2010;116:4906-4915.
68. Richardson PG, Spencer A, Cannell P, et al. Phase 1 Clinical Evaluation of Twice-Weekly Marizomib (NPI-0052), a Novel Proteasome Inhibitor, in Patients with Relapsed/Refractory Multiple Myeloma (MM). *ASH Annual Meeting Abstracts*. 2011;118:302.
69. Chauhan D, Catley L, Li G, et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from bortezomib. *Cancer Cell*. 2005;8:407-419.
70. Groll M, Potts BC. Proteasome structure, function, and lessons learned from beta-lactone inhibitors. *Curr Top Med Chem*. 2011;11:2850-2878.
72. Schneider C, Sepp-Lorenzino L, Nimmesgern E, et al. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc Natl Acad Sci U S A*. 1996;93:14536-14541.
77. Richardson PG, Badros AZ, Jagannath S, et al. Tanespimycin with bortezomib: activity in relapsed/refractory patients with multiple myeloma. *Br J Haematol*. 2010;150:428-437.
78. Richardson PG, Chanan-Khan AA, Alsina M, et al. Tanespimycin monotherapy in relapsed multiple myeloma: results of a phase 1 dose-escalation study. *Br J Haematol*. 2010;150:438-445.
79. Richardson PG, Chanan-Khan AA, Lonial S, et al. Tanespimycin and bortezomib combination treatment in patients with relapsed or relapsed and refractory multiple myeloma: results of a phase 1/2 study. *Br J Haematol*. 2011;153:729-740.
80. Richardson PG, Mitsiades CS, Laubach JP, et al. Inhibition of heat shock protein 90 (HSP90) as a therapeutic strategy for the treatment of myeloma and other cancers. *Br J Haematol*. 2011;152:367-379.
81. Ri M, Iida S, Nakashima T, et al. Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. *Leukemia*. 2010;24:1506-1512.
82. Nakashima T, Ishii T, Tagaya H, et al. New molecular and biological mechanism of antitumor activities of KW-2478, a novel nonansamycin heat shock protein 90 inhibitor, in multiple myeloma cells. *Clin Cancer Res*. 2010;16:2792-2802.
83. Usmani SZ, Bona RD, Chiosis G, Li Z. The anti-myeloma activity of a novel purine scaffold HSP90 inhibitor PU-H71 is via inhibition of both HSP90A and HSP90B1. *J Hematol Oncol*. 2010;3:40.
84. Mimnaugh EG, Xu W, Vos M, et al. Simultaneous inhibition of hsp 90 and the proteasome promotes protein ubiquitination, causes endoplasmic reticulum-derived cytosolic vacuolization, and enhances antitumor activity. *Mol Cancer Ther*. 2004;3:551-566.
85. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med*. 2004;10:1321-1328.
86. Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*. 2004;303:844-848.
87. Sumimoto H, Yamagata S, Shimizu A, et al. Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference. *Gene Ther*. 2005;12:95-100.
88. Wang G, Chan CH, Gao Y, Lin HK. Novel roles of Skp2 E3 ligase in cellular senescence, cancer progression, and metastasis. *Chin J Cancer*. 2012;31:169-177.
89. Banerji U, O'Donnell A, Scurr M, et al. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino,17-demethoxygeldanamycin in patients with advanced malignancies. *J Clin Oncol*. 2005;23:4152-4161.
90. Eiseman JL, Lan J, Lagattuta TF, et al. Pharmacokinetics and pharmacodynamics of 17-demethoxy 17-[[(2-dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts. *Cancer Chemother Pharmacol*. 2005;55:21-32.
91. Plescia J, Salz W, Xia F, et al. Rational design of shepherdin, a novel anticancer agent. *Cancer Cell*. 2005;7:457-468.
92. Vilenchik M, Solit D, Basso A, et al. Targeting wide-range oncogenic transformation via PU24FCl, a specific inhibitor of tumor Hsp90. *Chem Biol*. 2004;11:787-797.
93. Xu L, Eiseman JL, Egorin MJ, D'Argenio DZ. Physiologically-based pharmacokinetics and molecular pharmacodynamics of 17-(allylamino)-17-demethoxygeldanamycin and its active metabolite in tumor-bearing mice. *J Pharmacokinet Pharmacodyn*. 2003;30:185-219.
94. Chiosis G, Huezo H, Rosen N, et al. 17AAG: Low target binding affinity and potent cell activity-finding an explanation. *Mol Cancer Ther*. 2003;2:123-129.
95. Kamal A, Thao L, Sensintaffar J, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*. 2003;425:407-410.

96. Moulick K, Ahn JH, Zong H, et al. Affinity-based proteomics reveal cancer-specific networks coordinated by Hsp90. *Nat Chem Biol*. 2011;7:818-826.
98. Guo W, Reigan P, Siegel D, et al. Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition. *Cancer Res*. 2005;65:10006-10015.
100. Porter JR, Fritz CC, Depew KM. Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. *Curr Opin Chem Biol*. 2010;14:412-420.
101. Hanson BE, Vesole DH. Retaspimycin hydrochloride (IPI-504): a novel heat shock protein inhibitor as an anticancer agent. *Expert Opin Investig Drugs*. 2009;18:1375-1383.
102. Kisselev AF, Callard A, Goldberg AL. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem*. 2006;281:8582-8590.
109. Fumo G, Akin C, Metcalfe DD, Neckers L. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. *Blood*. 2004;103:1078-1084.
110. Grbovic OM, Basso A, Sawai A, et al. V600E B-Raf requires the Hsp90 chaperone for stability and is degraded in response to Hsp90 inhibitors. *Proc Natl Acad Sci U S A*. 2006;103:57-62.
111. da Rocha Dias S, Friedlos F, Light Y, et al. Activated B-RAF is an Hsp90 client protein that is targeted by the anticancer drug 17-allylamino-17-demethoxygeldanamycin. *Cancer Res*. 2005;65:10686-10691.
112. Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. *Cancer Res*. 2005;65:6401-6408.
119. Zagzag D, Nomura M, Friedlander DR, et al. Geldanamycin inhibits migration of glioma cells in vitro: A potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. *J Cell Physiol*. 2003;196:394-402.
122. Pennacchietti S, Michieli P, Galluzzo M, et al. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*. 2003;3:347-361.
124. Bottaro DP, Liotta LA. Out of air is not out of action. *Nature*. 2003;423:593-595.
127. Ichihara M, Murakumo Y, Takahashi M. RET and neuroendocrine tumors. *Cancer Lett*. 2004;204:197-211.

References

1. Stoler DL, Chen N, Basik M, et al. The onset and extent of genomic instability in sporadic colorectal tumor progression. *Proc Natl Acad Sci U S A*. 1999;96:15121-15126.
2. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. *Nat Rev Cancer*. 2002;2:331-341.
3. La Rosee P, O'Dwyer ME, Druker BJ. Insights from pre-clinical studies for new combination treatment regimens with the Bcr-Abl kinase inhibitor imatinib mesylate (Gleevec/Glivec) in chronic myelogenous leukemia: a translational perspective. *Leukemia*. 2002;16:1213-1219.
4. Kitano H. Cancer robustness: tumour tactics. *Nature*. 2003;426:125.
5. Hetz C, Soto C. Protein misfolding and disease: the case of prion disorders. *Cell Mol Life Sci*. 2003;60:133-143.
6. Nardai G, Vegh EM, Prohaszka Z, Csermely P. Chaperone-related immune dysfunction: an emergent property of distorted chaperone networks. *Trends Immunol*. 2006;27:74-79.
7. Zhao R, Davey M, Hsu YC, et al. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell*. 2005;120:715-727.
8. Sreedhar AS, Soti C, Csermely P. Inhibition of Hsp90: a new strategy for inhibiting protein kinases. *Biochim Biophys Acta*. 2004;1697:233-242.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
10. Isaacs JS. Heat-shock protein 90 inhibitors in antineoplastic therapy: is it all wrapped up? *Expert Opin Investig Drugs*. 2005;14:569-589.
11. Chiosis G, Vilenchik M, Kim J, Solit D. Hsp90: the vulnerable chaperone. *Drug Discov Today*. 2004;9:881-888.
12. Workman P. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. *Cancer Lett*. 2004;206:149-157.
13. Bagatell R, Whitesell L. Altered Hsp90 function in cancer: a unique therapeutic opportunity. *Mol Cancer Ther*. 2004;3:1021-1030.
14. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med*. 2004;82:488-499.
15. Goetz MP, Toft DO, Ames MM, Erlichman C. The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann Oncol*. 2003;14:1169-1176.
16. Isaacs JS, Xu W, Neckers L. Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell*. 2003;3:213-217.
17. Rutherford SL, Lindquist S. Hsp90 as a capacitor for morphological evolution. *Nature*. 1998;396:336-342.
18. Queitsch C, Sangster TA, Lindquist S. Hsp90 as a capacitor of phenotypic variation. *Nature*. 2002;417:618-624.
19. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer*. 2005;5:761-772.
20. Tang YC, Williams BR, Siegel JJ, Amon A. Identification of aneuploidy-selective antiproliferation compounds. *Cell*. 2011;144:499-512.
21. DeBoer C, Meulman PA, Wnuk RJ, Peterson DH. Geldanamycin, a new antibiotic. *J Antibiot (Tokyo)*. 1970;23:442-447.
22. Schulte TW, Neckers LM. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother Pharmacol*. 1998;42:273-279.
23. Porter JR, Ge J, Lee J, et al. Ansamycin inhibitors of Hsp90: nature's prototype for anti-chaperone therapy. *Curr Top Med Chem*. 2009;9:1386-1418.
24. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer*. 2010;10:537-549.
25. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? *Clin Cancer Res*. 2012;18:64-76.
26. Siligardi G, Hu B, Panaretou B, et al. Co-chaperone regulation of conformational switching in the Hsp90 ATPase cycle. *J Biol Chem*. 2004;279:51989-51998.
27. Wegele H, Muller L, Buchner J. Hsp70 and Hsp90—a relay team for protein folding. *Rev Physiol Biochem Pharmacol*. 2004;151:1-44.
28. Prodromou C, Pearl LH. Structure and functional relationships of Hsp90. *Curr Cancer Drug Targets*. 2003;3:301-323.
29. He H, Zatorska D, Kim J, et al. Identification of potent water soluble purine-scaffold inhibitors of the heat shock protein 90. *J Med Chem*. 2006;49:381-390.
30. Llauger L, He H, Kim J, et al. Evaluation of 8-arylsulfanyl, 8-arylsulfoxyl, and 8-arylsulfonyl adenine derivatives as inhibitors of the heat shock protein 90. *J Med Chem*. 2005;48:2892-2905.
31. Chiosis G, Lucas B, Huezo H, et al. Development of purine-scaffold small molecule inhibitors of Hsp90. *Curr Cancer Drug Targets*. 2003;3:371-376.
32. Chiosis G, Lucas B, Shtil A, et al. Development of a purine-scaffold novel class of Hsp90 binders that inhibit the proliferation of cancer cells and induce the degradation of Her2 tyrosine kinase. *Bioorg Med Chem*. 2002;10:3555-3564.
33. Cheung KM, Matthews TP, James K, et al. The identification, synthesis, protein crystal structure and in vitro biochemical evaluation of a new 3,4-diarylpyrazole class of Hsp90 inhibitors. *Bioorg Med Chem Lett*. 2005;15:3338-3343.
34. Dymock BW, Barril X, Brough PA, et al. Novel, potent small-molecule inhibitors of the molecular chaperone Hsp90 discovered through structure-based design. *J Med Chem*. 2005;48:4212-4215.
35. Kim YS, Alarcon SV, Lee S, et al. Update on Hsp90 inhibitors in clinical trial. *Curr Top Med Chem*. 2009;9:1479-1492.
36. Scheibel T, Buchner J. The Hsp90 complex–a super-chaperone machine as a novel drug target. *Biochem Pharmacol*. 1998;56:675-682.
37. Hernandez MP, Chadli A, Toft DO. HSP40 binding is the first step in the HSP90 chaperoning pathway for the progesterone receptor. *J Biol Chem*. 2002;277:11873-11881.
38. McLaughlin SH, Smith HW, Jackson SE. Stimulation of the weak ATPase activity of human hsp90 by a client protein. *J Mol Biol*. 2002;315:787-798.
39. Siligardi G, Panaretou B, Meyer P, et al. Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37. *J Biol Chem*. 2002;277:20151-20159.
40. Young JC, Hartl FU. Polypeptide release by Hsp90 involves ATP hydrolysis and is enhanced by the co-chaperone p23. *EMBO J*. 2000;19:5930-5940.
41. Panaretou B, Siligardi G, Meyer P, et al. Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. *Mol Cell*. 2002;10:1307-1318.
42. Xu W, Mollapour M, Prodromou C, et al. Dynamic tyrosine phosphorylation modulates cycling of the HSP90-P50(CDC37)-AHA1 chaperone machine. *Mol Cell*. 2012;47:434-443.
43. Mollapour M, Neckers L. Post-translational modifications of Hsp90 and their contributions to chaperone regulation. *Biochim Biophys Acta*. 2012;1823:648-655.
44. Mollapour M, Tsutsumi S, Donnelly AC, et al. Swe1Wee1-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function. *Mol Cell*. 2010;37:333-343.
45. Mollapour M, Tsutsumi S, Kim YS, et al. Casein kinase 2 phosphorylation of Hsp90 threonine 22 modulates chaperone function and drug sensitivity. *Oncotarget*. 2011;2:407-417.
46. Neckers L. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol Med*. 2002;8(4 suppl):S55-S61.
47. Xu W, Marcu M, Yuan X, et al. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc Natl Acad Sci U S A*. 2002;99:12847-12852.
48. Ehrlich ES, Wang T, Luo K, et al. Regulation of Hsp90 client proteins by a Cullin5-RING E3 ubiquitin ligase. *Proc Natl Acad Sci U S A*. 2009;106:20330-20335.
49. An WG, Schulte TW, Neckers LM. The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome. *Cell Growth Differ*. 2000;11:355-360.
50. Mollapour M, Tsutsumi S, Neckers L. Hsp90 phosphorylation, Wee1 and the cell cycle. *Cell Cycle*. 2010;9:2310-2316.
51. Stommel JM, Kimmelman AC, Ying H, et al. Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies. *Science*. 2007;318:287-290.
52. Bachleitner-Hofmann T, Sun MY, Chen CT, et al. HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells. *Mol Cancer Ther*. 2008;7:3499-3508.
53. Pashtan I, Tsutsumi S, Wang S, et al. Targeting Hsp90 prevents escape of breast cancer cells from tyrosine kinase inhibition. *Cell Cycle*. 2008;7:2936-2941.

54. Wang S, Pashtan I, Tsutsumi S, et al. Cancer cells harboring MET gene amplification activate alternative signaling pathways to escape MET inhibition but remain sensitive to Hsp90 inhibitors. *Cell Cycle*. 2009;8:2050-2056.
55. Koizumi H, Yamada T, Takeuchi S, et al. Hsp90 inhibition overcomes HGF-triggering resistance to EGFR-TKIs in EGFR-mutant lung cancer by decreasing client protein expression and angiogenesis. *J Thorac Oncol*. 2012;7:1078-1085.
56. Goldberg AL. Functions of the proteasome: From protein degradation and immune surveillance to cancer therapy. *Biochem Soc Trans*. 2007;35:12-17.
57. Nalepa G, Rolfe M, Harper JW. Drug discovery in the ubiquitin-proteasome system. *Nat Rev Drug Discov*. 2006;5:596-613.
58. Reinstein E, Ciechanover A. Protein degradation and human diseases: the ubiquitin connection. *Ann Intern Med*. 2006;145:676-207.
59. Roccaro AM, Hideshima T, Richardson PG, et al. Bortezomib as an antitumor agent. *Curr Pharm Biotech*. 2006;7:441-448.
60. Moreau P, Richardson PG, Cavo M, et al. Proteasome inhibitors in multiple myeloma: ten years later. *Blood*. 2012;120:947-959.
61. Kupperman E, Lee EC, Cao Y, et al. Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res*. 2010;70:1970-1980.
62. Richardson PG, Baz R, Wang L, et al. Investigational agent MLN9708, an oral proteasome inhibitor, in patients (pts) with relapsed and/or refractory multiple myeloma (MM): results from the expansion cohorts of a phase 1 dose-escalation study. *ASH Annual Meeting Abstracts*. 2011;118:301.
63. Piva R, Ruggeri B, Williams M, et al. CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. *Blood*. 2008;111:2765-2775.
64. Demo SD, Kirk CJ, Aujay MA, et al. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res*. 2007;67:6383-6391.
65. Kuhn DJ, Chen Q, Voorhees PM, et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood*. 2007;110:3281-3290.
66. Jain S, Diefenbach C, Zain J, O'Connor OA. Emerging role of carfilzomib in treatment of relapsed and refractory lymphoid neoplasms and multiple myeloma. *Core Evid*. 2011;6:43-57.
67. Chauhan D, Singh AV, Aujay M, et al. A novel orally active proteasome inhibitor ONX 0912 triggers in vitro and in vivo cytotoxicity in multiple myeloma. *Blood*. 2010;116:4906-4915.
68. Richardson PG, Spencer A, Cannell P, et al. Phase 1 Clinical Evaluation of Twice-Weekly Marizomib (NPI-0052), a Novel Proteasome Inhibitor, in Patients with Relapsed/Refractory Multiple Myeloma (MM). *ASH Annual Meeting Abstracts*. 2011;118:302.
69. Chauhan D, Catley L, Li G, et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from bortezomib. *Cancer Cell*. 2005;8:407-419.
70. Groll M, Potts BC. Proteasome structure, function, and lessons learned from beta-lactone inhibitors. *Curr Top Med Chem*. 2011;11:2850-2878.
71. Mimnaugh EG, Chavany C, Neckers L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J Biol Chem*. 1996;271:22796-22801.
72. Schneider C, Sepp-Lorenzino L, Nimmesgern E, et al. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc Natl Acad Sci U S A*. 1996;93:14536-14541.
73. Basso AD, Solit DB, Chiosis G, et al. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem*. 2002;277:39858-39866.
74. French BA, van Leeuwen F, Riley NE, et al. Aggresome formation in liver cells in response to different toxic mechanisms: role of the ubiquitin-proteasome pathway and the frameshift mutant of ubiquitin. *Exp Mol Pathol*. 2001;71:241-246.
75. Waelter S, Boeddrich A, Lurz R, et al. Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol Biol Cell*. 2001;12:1393-1407.
76. Mitsiades N, Mitsiades CS, Poulaki V, et al. Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci U S A*. 2002;99:14374-14379.
77. Richardson PG, Badros AZ, Jagannath S, et al. Tanespimycin with bortezomib: activity in relapsed/refractory patients with multiple myeloma. *Br J Haematol*. 2010;150:428-437.
78. Richardson PG, Chanan-Khan AA, Alsina M, et al. Tanespimycin monotherapy in relapsed multiple myeloma: results of a phase 1 dose-escalation study. *Br J Haematol*. 2010;150:438-445.
79. Richardson PG, Chanan-Khan AA, Lonial S, et al. Tanespimycin and bortezomib combination treatment in patients with relapsed or relapsed and refractory multiple myeloma: results of a phase 1/2 study. *Br J Haematol*. 2011;153:729-740.
80. Richardson PG, Mitsiades CS, Laubach JP, et al. Inhibition of heat shock protein 90 (HSP90) as a therapeutic strategy for the treatment of myeloma and other cancers. *Br J Haematol*. 2011;152:367-379.
81. Ri M, Iida S, Nakashima T, et al. Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. *Leukemia*. 2010;24:1506-1512.
82. Nakashima T, Ishii T, Tagaya H, et al. New molecular and biological mechanism of antitumor activities of KW-2478, a novel nonansamycin heat shock protein 90 inhibitor, in multiple myeloma cells. *Clin Cancer Res*. 2010;16:2792-2802.
83. Usmani SZ, Bona RD, Chiosis G, Li Z. The anti-myeloma activity of a novel purine scaffold HSP90 inhibitor PU-H71 is via inhibition of both HSP90A and HSP90B1. *J Hematol Oncol*. 2010;3:40.
84. Mimnaugh EG, Xu W, Vos M, et al. Simultaneous inhibition of hsp 90 and the proteasome promotes protein ubiquitination, causes endoplasmic reticulum-derived cytosolic vacuolization, and enhances antitumor activity. *Mol Cancer Ther*. 2004;3:551-566.
85. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med*. 2004;10:1321-1328.
86. Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science*. 2004;303:844-848.
87. Sumimoto H, Yamagata S, Shimizu A, et al. Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference. *Gene Ther*. 2005;12:95-100.
88. Wang G, Chan CH, Gao Y, Lin HK. Novel roles of Skp2 E3 ligase in cellular senescence, cancer progression, and metastasis. *Chin J Cancer*. 2012;31:169-177.
89. Banerji U, O'Donnell A, Scurr M, et al. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino,17-demethoxygeldanamycin in patients with advanced malignancies. *J Clin Oncol*. 2005;23:4152-4161.
90. Eiseman JL, Lan J, Lagattuta TF, et al. Pharmacokinetics and pharmacodynamics of 17-demethoxy 17-[[(2-dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts. *Cancer Chemother Pharmacol*. 2005;55:21-32.
91. Plescia J, Salz W, Xia F, et al. Rational design of shepherdin, a novel anticancer agent. *Cancer Cell*. 2005;7:457-468.
92. Vilenchik M, Solit D, Basso A, et al. Targeting wide-range oncogenic transformation via PU24FCl, a specific inhibitor of tumor Hsp90. *Chem Biol*. 2004;11:787-797.
93. Xu L, Eiseman JL, Egorin MJ, D'Argenio DZ. Physiologically-based pharmacokinetics and molecular pharmacodynamics of 17-(allylamino)-17-demethoxygeldanamycin and its active metabolite in tumor-bearing mice. *J Pharmacokinet Pharmacodyn*. 2003;30:185-219.
94. Chiosis G, Huezo H, Rosen N, et al. 17AAG: Low target binding affinity and potent cell activity-finding an explanation. *Mol Cancer Ther*. 2003;2:123-129.
95. Kamal A, Thao L, Sensintaffar J, et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*. 2003;425:407-410.
96. Moulick K, Ahn JH, Zong H, et al. Affinity-based proteomics reveal cancer-specific networks coordinated by Hsp90. *Nat Chem Biol*. 2011;7:818-826.
97. Kelland LR, Sharp SY, Rogers PM, et al. DT-Diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90. *J Natl Cancer Inst*. 1999;91:1940-1949.
98. Guo W, Reigan P, Siegel D, et al. Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition. *Cancer Res*. 2005;65:10006-10015.
99. Belinsky M, Jaiswal AK. NAD(P)H:quinone oxidoreductase1 (DT-diaphorase) expression in normal and tumor tissues. *Cancer Metastasis Rev*. 1993;12:103-117.

100. Porter JR, Fritz CC, Depew KM. Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. *Curr Opin Chem Biol*. 2010;14:412-420.
101. Hanson BE, Vesole DH. Retaspimycin hydrochloride (IPI-504): a novel heat shock protein inhibitor as an anticancer agent. *Expert Opin Investig Drugs*. 2009;18:1375-1383.
102. Kisselev AF, Callard A, Goldberg AL. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem*. 2006;281:8582-8590.
103. Fujimoto J, Shiota M, Iwahara T, et al. Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). *Proc Natl Acad Sci U S A*. 1996;93:4181-4186.
104. Bonvini P, Gastaldi T, Falini B, Rosolen A. Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), a novel Hsp90-client tyrosine kinase: down-regulation of NPM-ALK expression and tyrosine phosphorylation in ALK$^+$ CD30$^+$ lymphoma cells by the Hsp90 antagonist 17-allylamino,17-demethoxygeldanamycin. *Cancer Res*. 2002;62:1559-1566.
105. Naoe T, Kiyoe H, Yamamoto Y, et al. FLT3 tyrosine kinase as a target molecule for selective antileukemia therapy. *Cancer Chemother Pharmacol*. 2001;48(suppl 1):S27-S30.
106. Minami Y, Kiyoi H, Yamamoto Y, et al. Selective apoptosis of tandemly duplicated FLT3-transformed leukemia cells by Hsp90 inhibitors. *Leukemia*. 2002;16:1535-1540.
107. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*. 2002;99:3530-3539.
108. Shah NP, Nicoll JM, Nagar B, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell*. 2002;2:117-125.
109. Fumo G, Akin C, Metcalfe DD, Neckers L. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. *Blood*. 2004;103:1078-1084.
110. Grbovic OM, Basso A, Sawai A, et al. V600E B-Raf requires the Hsp90 chaperone for stability and is degraded in response to Hsp90 inhibitors. *Proc Natl Acad Sci U S A*. 2006;103:57-62.
111. da Rocha Dias S, Friedlos F, Light Y, et al. Activated B-RAF is an Hsp90 client protein that is targeted by the anticancer drug 17-allylamino-17-demethoxygeldanamycin. *Cancer Res*. 2005;65:10686-10691.
112. Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. *Cancer Res*. 2005;65:6401-6408.
113. Rajagopalan H, Bardelli A, Lengauer C, et al. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature*. 2002;418:934.
114. Vanaja DK, Mitchell SH, Toft DO, Young CYF. Effect of geldanamycin on androgen receptor function and stability. *Cell Stress Chaperones*. 2002;7:55-64.
115. Georget V, Terouanne B, Nicolas J-C, Sultan C. Mechanism of antiandrogen action: Key role of Hsp90 in conformational change and transcriptional activity of the androgen receptor. *Biochemistry*. 2002;41:11824-11831.
116. Solit D, Zheng F, Drobnjak M, et al. 17-allylamino-17-demthoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clin Cancer Res*. 2002:986-993.
117. Isaacs JS, Jung YJ, Mimnaugh EG, et al. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. *J Biol Chem*. 2002;277:29936-29944.
118. Mabjeesh NJ, Post DE, Willard MT, et al. Geldanamycin induces degradation of hypoxia-inducible factor 1a protein via the proteasome pathway in prostate cancer cells. *Cancer Res*. 2002;62:2478-2482.
119. Zagzag D, Nomura M, Friedlander DR, et al. Geldanamycin inhibits migration of glioma cells in vitro: A potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. *J Cell Physiol*. 2003;196:394-402.
120. Dias S, Shmelkov SV, Lam G, Rafii S. VEGF promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. *Blood*. 2002;99:2532-2540.
121. Maulik G, Kijima T, Ma PC, et al. Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. *Clin Cancer Res*. 2002;8:620-627.
122. Pennacchietti S, Michieli P, Galluzzo M, et al. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell*. 2003;3:347-361.
123. Tacchini L, Dansi P, Matteucci E, Desiderio MA. Hepatocyte growth factor signalling stimulates hypoxia inducible factor-1 (HIF-1) activity in HepG2 hepatoma cells. *Carcinogenesis*. 2001;22:1363-1371.
124. Bottaro DP, Liotta LA. Out of air is not out of action. *Nature*. 2003;423:593-595.
125. Santoro M, Melillo RM, Carlomagno F, et al. Molecular mechanisms of RET activation in human cancer. *Ann N Y Acad Sci*. 2002;963:116-121.
126. Jhiang SM. The RET proto-oncogene in human cancers. *Oncogene*. 2000;19:5590-5597.
127. Ichihara M, Murakumo Y, Takahashi M. RET and neuroendocrine tumors. *Cancer Lett*. 2004;204:197-211.
128. Cohen MS, Hussain HB, Moley JF. Inhibition of medullary thyroid carcinoma cell proliferation and RET phosphorylation by tyrosine kinase inhibitors. *Surgery*. 2002;132:960-966; discussion 966–967.

Suhendan Ekmekcioglu, Razelle Kurzrock, and Elizabeth A. Grimm

57

Hematopoietic Growth Factors and Cytokines

Growth factors regulate the essential cellular process of proliferation and differentiation. Overproduction of growth factors is a common feature of tumor cells, stimulating unregulated proliferation of themselves in an autocrine fashion, and of adjacent cells in a paracrine fashion. More specifically, hematopoietic growth factor is a common term for the family of glycoproteins that regulate proliferation and differentiation of hematopoietic cells. Cytokines are a subtype of growth factors that are produced by hematopoietic and immune cell types and include interferons and interleukins. The term *cytokine* refers to a chemical messenger protein that carries a biochemical signal between cells, usually of the immune system, and the rest of the body. *Interleukin* designates any soluble protein or glycoprotein product of leukocytes that regulates the responses of other leukocytes. The pleiotropic nature of many cytokines and interleukins allows them to influence virtually all organ systems. In addition to their vital role in promoting hematopoietic cell growth, differentiation, and activity, these molecules are vital to the proper functioning of the central nervous system, cardiorespiratory system, and liver, as well as to bone remodeling, lipid metabolism, and embryogenesis and maintenance of pregnancy. Interestingly, many of these molecules have pleiotropic effects on numerous organ systems. For example, stem cell factor influences hematopoiesis and neurogenesis, and prolactin promotes multiproduction and erythropoiesis. Cytokines may have their own private receptor but may also share a "public" receptor with other cytokines (Table 57-1[1,2]), perhaps explaining some of the redundancy in their effects (Figure 57-1).

The identification and cloning of hematopoietic growth factors and cytokines have revolutionized medical practice. Raising white blood cell counts in patients with neutropenia was unimaginable until the discovery of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Today, growth factors are routinely used to alleviate neutropenia and, to a lesser extent, thrombocytopenia and anemia after chemotherapy. They can also help mobilize stem cells for transplantation, and they may have the potential to mobilize the immune system against infection or cancer.

Herein, we give an overview of the biologic characterization of the known clinically relevant interleukins and selected cytokines, the rationale for their use in therapy for patients with cancer, and the clinical experience with them.

Erythropoietin

Erythropoietin is produced by juxtaglomerular cells of the kidney. It is the most important hormone regulator of erythropoiesis. It has an accepted place in the treatment of anemia caused by a variety of illnesses (Table 57-2[3]). Because its primary production source is the kidney, it is not surprising that EPO has its best established role in the treatment of anemia due to the EPO-deficient state in kidney disease. However, many patients with anemia due to cancer also have a relative deficiency in endogenous EPO and respond to EPO. Interestingly, certain cases of familial erythrocytosis have been attributed to the presence of EPO-hypersensitive cells. This heightened EPO response results from the formation of a truncated EPO receptor that is missing a negative regulatory domain.[1]

EPO: Clinical Trials/Applications

EPO is most useful in those anemias in which there is an absolute or a relative deficiency in endogenous EPO levels, such as in renal failure and cancer, respectively (see Table 57-2). The quality of life for patients with anemia due to renal failure or cancer who respond to EPO is clearly improved. Although EPO has generally been used for patients with hemoglobin below 10 g/dL, the maximum improvement in the quality of life actually

Table 57-1 Receptors, Natural Antagonists, and Chromosomal Locations of Growth Factors and Cytokines

	Receptor	Natural Antagonists	Chromosomal Locations
Erythropoietin (EPO) (136)	EPO receptor	Soluble EPO receptor	7q21
GM-CSF	Type I receptor with α and β subunits		5q31.1
G-CSF	G-CSF receptor		17q11.2-q12
M-CSF	Fms		1p21-p13
Stem cell factor	c-kit	Soluble c-kit receptor	12q22-12q24
Thrombopoietin	Mp1		3q27-q28
IL-1	IL-1RI and IL-1RII? Extended family of 10 members including IL-18R	Soluble IL-1RI and IL-1RII and IL-1RA	2q13
IL-2	$\alpha\beta\gamma$ heterotrimeric complex		4q26-q27
IL-3	IL-3 receptor (heterodimer of IL-3 specific α subunit and β subunit)		5q31
IL-4 and IL-13	IL-4 and IL-13 receptors share subunits Type 1 IL-4 receptor (IL-4Rα and IL-2 receptor γc chain subunits) transduces IL-4; type II IL-4 receptor (IL-4Rα and the IL-13Rα1 subunits) transduces IL-4 and IL-13; IL-4Rα and IL-13Rα2 complex or two IL-13Rα transduce IL-13	Soluble IL-4 and IL-13 receptors exist	5q31
IL-5	Consists of IL-5Rα (IL-5-specific) and a β subunit. β subunit is common to IL-3 and GM-CSF complexes		5q31
IL-6	IL-6Rα together with gp130		7p21
IL-7	Composed of IL-7Rα (CD127) and the common γc chain subunits		8q12-q13
IL-8	IL-8Rα and IL-8Rβ exist		4q12-q13
IL-9	IL-9 receptor		5q31.1
IL-10	IL-10 receptor interferon receptors		1q31-q32
IL-11	IL-11Rα and gp 130 subunits gp 130 = CD130 on 5q11 IL-6, oncostatin M, and leukemia inhibitory factor also use gp130 subunit		19q13.3-q13.4
IL-12	IL-12Rβ1 and IL-12Rβ2 chains are related to gp 130	IL-12 p40 homodimers	IL-12A:3p12-q13.2 IL-12B:5q31.1-q33.1
IL-15	High-affinity receptor requires IL-2Rβ and γ chains and IL-15Rα chain		4q31
IL-16	Requires CD4 for biologic activities		15q26.1
IL-17	IL-17 receptor		2q31
IL-18	IL-18 receptor	IL-18 binding protein exists	11q22.2-q22.3
IL-19	IL-20Rl and IL-20R2		1q32
IL-20	IL-20R1 and IL-20R2		1q32
IL-21	IL-21 receptor		4q26–27
IL-22	IL-22R1 and IL-10R2		12q14
IL-23	IL-12Rb1 and IL-23R		12q13
IL-24	IL-20R1 and IL-20R2 IL-22R1 and IL-20R2		1q32
IL-25	IL-17BR		14q11
IL-26	IL-20R1 and IL-10R2		12q14
IL-27	TCCR/WSX-1 and GP130		12q13

Table 57-1 Receptors, Natural Antagonists, and Chromosomal Locations of Growth Factors and Cytokines—cont'd

	Receptor	Natural Antagonists	Chromosomal Locations
IL-28A, 28B, and 29	IL-28R1 and IL-10R2		19q13
IL-31	IL-31 receptor A and oncostatin M receptor		12q24
IL-32	Proteinase 3		16p13.3
IL-33	ST2		9p24.1
IL-35	IL-12Rβ2 and gp130		3 E 1
IL-36	IL-1Rrp2 and IL-1RAcP		IL36A:2q12-q14.1 IL36B:2q14 IL36G:2q12-q21 IL36RN:2q14
IL-37	IL-18R		2q12-q14.1
IL-38	IL36R		2q13

G-CSF, Granulocyte colony-stimulating factor; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *IL,* interleukin; *M-CSF,* macrophage colony-stimulating factor.

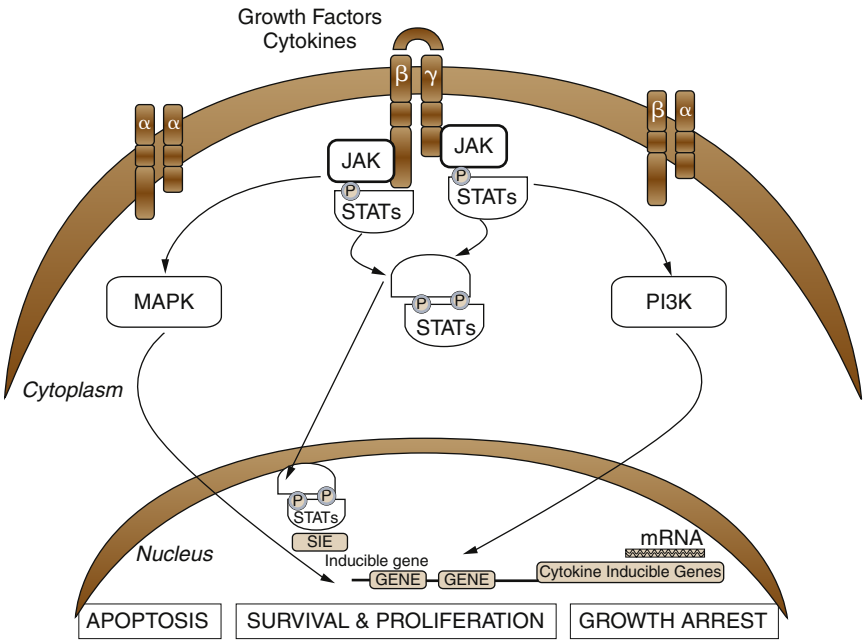


Figure 57-1 Actions of cytokine/growth factors binding to their receptors on the cell surface Cytokines and growth factors are the messengers that mediate intercellular communication. The regulation of cellular and nuclear functions by cytokines and growth factors is initiated through the activation of cell surface receptors. All receptors have a ligand-binding domain that ensures ligand specificity and an effector domain that initiates the generation of the biologic response on ligand binding. The activated receptor may then interact with other cellular components to complete the signal transduction process. Briefly, cytokine binding to receptor subunits induces homo- or heterodimerization resulting in the activation of Jaks that are bound to the receptor chains. The Jaks in turn phosphorylate tyrosine-based docking sites on the receptor. STATs bind via their SH2 domains. The STATs are then phosphorylated, form homo- or heterodimers, and translocate to the nucleus, where they bind target sequences, thereby regulating gene expression.

occurs in patients with higher hemoglobin levels (11 to 13 g/dL[1]). In the case of cancer, however, not all patients respond, and those with the highest levels of endogenous EPO are probably less likely to benefit. A recently discovered complicating factor to defining optimal EPO treatment has been the finding of decreased survival in some patients treated in randomized trials with "optimization" of hemoglobin.[4]

Erythropoietin-stimulating agents (ESAs) have been shown in clinical trials to decrease the transfusion requirements and increase the hemoglobin in patients with chemotherapy-induced anemia.[5-7] However, these trials have not shown that ESAs prolong survival or improve quality of life in these patients.[8] Moreover, ESAs have been associated with a number of unwanted outcomes in cancer patients, including an increased risk of stroke and venous thromboembolism, worse cancer outcomes, and increased mortality. With these findings of worse outcomes in patients treated with ESAs, the U.S. Food and Drug Administration (FDA) issued warnings against the use of ESA. The 2010 ASH/ASCO (American Society of Hematology/American Society of Clinical Oncology) Guidelines recommend a thorough workup for other causes of anemia before initiation of ESAs as well as discussion of the potential benefits and harms of ESAs.[7]

Table 57-2 Growth Factors and Cytokines in the Clinic*

Major Clinical Trials	
Erythropoietin (EPO) (136)	• Anemia of renal failure • Anemia of zidovudine therapy of HIV (with endogenous EPO level <500 mU/mL) • Anemia of cancer, especially after chemotherapy of solid tumors • Reduction of blood transfusions in elective surgery • Potentiation of autologous blood donation • Anemia of prematurity • Maximum quality-of-life improvement is at hemoglobin of 11-13 g/dL • Hyperglycosylated EPO (darbopoietin alfa) has prolonged half-life and can be administered less frequently • Postulated to offer neuroprotection after neurologic damage since EPO/EOP receptors are present in the central nervous system
GM-CSF	• Neutropenia due to myelosuppressive chemotherapy or bone marrow transplantation (BMT) • Peripheral blood stem cell mobilization • Graft failure • After induction therapy for acute myelocytic leukemia (AML)
G-CSF	• Neutropenia due to chemotherapy or BMT • Chronic and cyclic neutropenia • AIDS-related neutropenia • Autoimmune neutropenia • Peripheral blood stem cell mobilization • G-CSF reduces morbidity from high-risk febrile neutropenia treated with antibiotics
M-CSF	• Enhances hematopoietic recovery after chemotherapy or transplantation • Attenuates neutropenia in chronic neutropenia • Lowers serum cholesterol • May be useful in therapy of fungal infections
Stem cell factor (SCF)	• Peripheral blood progenitor mobilization (SCF + G-CSF better than SCF alone) • Aplastic anemia (trilineage responses seen after SCF)
Thrombopoietin	• Accelerates platelet recovery after chemotherapy • Increases platelet yield from normal donors for platelet transfusions • Enhances mobilization of peripheral blood progenitor cells by G-CSF • Nonimpressive effects on platelet recovery after myeloablative therapy
IL-1	IL-1α and IL-1β • Modest reduction in postchemotherapy neutropenia or thrombocytopenia; numerous side effects • No significant antitumor activity in melanoma or renal cell carcinoma IL-1RA • No clear-cut reduction in mortality in sepsis patients • Amelioration of rheumatoid arthritis and graft-versus-host disease
IL-2	• Antitumor activity in melanoma and renal cell carcinoma • IL-2 diphtheria fusion toxin (DAB-IL-2) approved for used in cutaneous T-cell lymphomas
IL-3	• Increases stem cell mobilization when used with G-CSF or GM-CSF • In combination with GM-CSF, hastens bone marrow recovery after transplant • Sequential IL-3 and GM-CSF produces multilineage responses in some marrow failure patients • Induces occasional sustained remissions in Diamond-Blackfan anemia
IL-4 and IL-13	• Only minor antitumor activity has been seen in a variety of human cancers of IL-4
IL-5	• IL-5 antagonists may be useful in the treatment of allergy and asthma. However, a trial of monoclonal antibody against IL-5 was not effective in asthma
IL-6	• Response rates 8%-14% in melanoma and renal cell carcinoma • Modest platelet-enhancing ability after chemotherapy or autologous transplant with significant toxicity • Antibody to block IL-6 is entering clinical trial
IL-8	• Antibodies to block IL-8 are entering clinical trial
IL-10	• Trend toward efficacy in rheumatoid arthritis, inflammatory bowel disease, and autoimmune diseases
IL-11	• Approved for use to prevent chemotherapy-induced thrombocytopenia
IL-12	• Potential use in vaccine development • No benefit in hepatitis C trial • Modest antitumor activity with significant toxicity in melanoma and renal cell carcinoma
IL-16	• May have potential use in HIV infection
IL-20	• May have potential for chronic inflammatory skin disease

Table 57-2 Growth Factors and Cytokines in the Clinic*—cont'd

Major Clinical Trials—cont'd	
IL-21	• Because it controls adaptive immune responses, its use in a clinical setting may prove efficacious for the treatment of cancer and infectious disease
IL-24	• Clinical trials for the treatment of melanoma are under way
IL-25	• Potent inflammatory activity and its association with various human disease states suggest this cytokine family as an important contributor to the pathophysiology of pulmonary diseases
IL-28A, 28B, and 29	• Alternative therapeutic choice to type 1 IFNs
IL-32	• Potential therapeutic target in rheumatoid arthritis

AIDS, Acquired immunodeficiency syndrome; *EOP*, erythropoietin; *G-CSF*, granulocyte colony-stimulating factor; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *M-CSF*, macrophage colony-stimulating factor; *HIV*, human immunodeficiency virus; *IFN*, interferon; *IL*, interleukin.
*Many of the listed applications refer to clinical trials and are not approved uses.

Granulocyte-Macrophage Colony-Stimulating Factor

Two hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), regulate the production and deployment of neutrophils. Both CSFs stimulate cell division and accelerate marrow transit times. Autonomous production by the tumor of GM-CSF (or G-CSF) has also been implicated as a pathophysiologic mechanism underlying leukemoid reactions in cancer patients. GM-CSF is used clinically for the treatment of neutropenia after chemotherapy or transplantation, for the treatment of graft failure, and for peripheral blood stem cell mobilization (see Table 57-2[9,10]).

GM-CSF: Clinical Trials/Applications

GM-CSF is safe and effective in the treatment of patients with acute myelogenous leukemia (AML) who are undergoing induction therapy. GM-CSF decreases the neutropenic period and the rate of serious infections in the elderly. This molecule is also indicated for accelerating myeloid reconstitution after allogeneic bone marrow transplantation. It also increases survival in patients who have engraftment failure or delay after allogeneic or autologous transplantation, and it can be exploited to enhance stem cell mobilization for transplant.

Granulocyte Colony-Stimulating Factor

G-CSF has revolutionized the treatment of neutropenia and its sequelae (infection). It has been used by millions of patients worldwide and is remarkably effective and virtually devoid of side effects (see Table 57-2[11]). Some patients with solid tumors present with significantly increased leukocyte counts due to G-CSF secretion. Finally, point mutations in the gene for the G-CSF receptor have been described anecdotally in patients with AML that evolved from severe congenital neutropenia.

G-CSF: Clinical Trials/Applications

G-CSF promotes a rapid increase in neutrophilic leukocytes, which lasts about 24 hours. Despite the multitude of patients who have received G-CSF, few side effects have been reported. Even very long-term G-CSF administration seems fairly innocuous; the most common toxicity is bone pain.

A summary of current American Society for Clinical Oncology (ASCO) guidelines suggests that CSF can be used for primary prophylaxis of febrile neutropenia after chemotherapy if the risk is about 20%. It is also recommended for patients at high risk, based on age, medical condition, disease characteristics, and myelotoxicity of chemotherapy. Prophylaxis is also recommended for diffuse aggressive lymphoma in patients older than 65 treated with curative therapy. Patients exposed to lethal radiotherapy should also receive CSF.[12,13] In the transplantation setting, the administration of G-CSF reduces neutropenia and infection. G-CSF also mobilizes autologous peripheral blood progenitor cells; these cells are used to accelerate hematopoietic recovery in patients who have received myeloablative or myelosuppressive chemotherapy. Finally, in patients with acquired immunodeficiency syndrome (AIDS), G-CSF reverses and prevents zidovudine-induced neutropenia.[11] Of interest, G-CSF may also be useful in enhancing the defenses of nonneutropenic patients with AIDS who have bacterial infections. However, studies have shown only modest benefit for G-CSF in the setting of nonneutropenic infection in normal individuals.

Macrophage Colony-Stimulating Factor

M-CSF affects a variety of organ systems, but its cardinal effect remains its ability to influence most aspects of monocyte/macrophage development and function (Table 57-3[14]). In addition to its hematopoietic effects, M-CSF and Fms (the M-CSF receptor) are expressed in the brain. This cytokine induces microglial proliferation, activation, and survival. In malignancy, mutations in Fms have been reported at codon 969 in about 10% of cases of human myeloid malignancies.

M-CSF: Clinical Trials/Applications

M-CSF given to patients with AML after consolidation chemotherapies shortened the periods of neutropenia and thrombocytopenia after chemotherapy and reduced the incidence and shortened the duration of febrile neutropenia.[14] Similar salutary effects have been reported after chemotherapy or bone marrow transplantation. M-CSF can elevate neutrophil counts in children with chronic neutropenia.

Stem-Cell Factor

Stem-cell factor (SCF) is also known as kit ligand, mast cell growth factor, or steel factor. It functions as a hematopoietic cytokine that triggers its biologic effect by binding to c-kit (the SCF receptor; see Table 57-3[15-18]). The average concentration of SCF in normal human serum is 3.3 ng/mL. Serum SCF concentrations are not elevated in patients with aplastic anemia, myelodysplasia, or chronic anemia or after marrow ablative therapy. Thus, the level of SCF in the circulation, unlike the level of EPO, is not inversely related to the number of hematopoietic cells. Alterations in the local distribution of SCF within the skin have been implicated in the pathogenesis of cutaneous mastocytosis.[1] Point mutations in the c-kit receptor cytoplasmic domain have been identified in murine and human mast cell lines and in hematopoietic cells from patients with mast-cell disorders. Finally, activating mutations in kit, a kinase receptor, characterize a type of leiomyosarcoma known as gastrointestinal stromal tumor. This finding has led to new targeted therapies of tremendous impact.

SCF: Clinical Trials/Applications

SCF seems to be reasonably well tolerated by patients, with the predominant side effects being transient local erythema and long-lasting hyperpigmentation at injection sites. The most worrisome toxicity is a mast cell effect resulting in allergic-like reactions characterized by urticaria, with or without respiratory symptoms.[1]

Of special interest is the role of mutations in the SCF receptor (kit) in gastrointestinal stromal tumors. These mutations activate the kinase enzymatic activity of kit. Kinase inhibitors targeted against kit (imatinib and sunitinib) have been found to be dramatically effective in these notoriously chemotherapy-resistant tumors.[16]

Thrombopoietin

The cytokine basis of megakaryocyte and platelet production has been more enigmatic than that of other lineages (see Table 57-3[19-26]). Factors that have now been implicated in at least some aspects of thrombocyte development include interleukin-3 (IL-3), IL-6, IL-9, IL-11, G-CSF, GM-CSF, SCF, leukemia inhibiting factor, and thrombopoietin (TPO). The last molecule is believed to be of paramount importance in the physiologic regulation of platelet production. Unfortunately, however, compared with the striking effects of the granulopoietic factors in neutropenic patients, use of the thrombopoietic molecules in the clinic setting has been disappointing. It has been suggested that the temporal pace of the thrombopoietic response is physiologically ordained to be considerably slower than the myelopoietic response, and that may explain why short courses of thrombopoietins seem to be ineffective.[13]

TPO: Clinical Trials/Applications

Two forms of TPO have entered clinical trials[20]: (1) TPO (the full-length polypeptide) and (2) polyethylene glycol (PEG)-conjugated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). Because its biologic action is prolonged, parenteral administration of TPO for 7 to 10 days increases platelet production 6 to 16 days later.[21] Results of clinical trials of PEG-rHuMGDF or recombinant human TPO in patients with cancer who were receiving chemotherapy, albeit with regimens that produce only moderate thrombocytopenia, suggest that platelet counts return to baseline significantly faster and that the nadir platelet counts are higher.[22] However, the effectiveness of these molecules in accelerating platelet recovery after myeloablative therapy has not been impressive.[23] Furthermore, in most patients with delayed platelet recovery after peripheral-blood stem-cell or bone marrow transplantation, recombinant human TPO did not significantly raise platelet counts.[24] TPO can result in

Table 57-3 Major Biologic Activities of Growth Factors and Cytokines

	Biologic Activities
Erythropoietin (136)	• Promotes the proliferation, differentiation, and survival of erythroid precursors
GM-CSF	• Stimulates growth of multilineage progenitors, BFU-E, granulocyte, macrophage, and eosinophil colonies • Induces migration and proliferation of vascular endothelial cells • Activates mature phagocytes (neutrophils, macrophages, eosinophils)
G-CSF	• Regulates production and function of neutrophils
M-CSF	• Influences most aspects of monocyte/macrophage development and function • Stimulates hematopoiesis • Induces osteoclast production • Helps maintain pregnancies • Lowers cholesterol levels • Affects microglial function
Stem cell factor	• Promotes hematopoiesis at multiple levels • Influences primordial germ cell and melanocyte migration during embryonic life • Affects immunoregulatory cells (B and T cells, mast cells, natural killer [NK] cells, dendritic cells) • Influences hematopoietic cell adhesive properties
Thrombopoietin	• Major regulator of platelet production • Acts in synergy with EPO to stimulate growth of erythroid progenitors • Acts in synergy with IL-3 and SCF to stimulate proliferation and prolong survival of hematopoietic stem cells
IL-1	• Induces production of multiple cytokines • Upregulates cell-surface cytokine expression • Synergizes with other cytokines to stimulate hematopoietic progenitor proliferation • Influences immune regulation (T- and B-cell responses) • Modulates endocrine function • Affects bone formation • IL-1 R acts as a cofactor in neural transmission • IL-1 is probably not critical for normal hematopoiesis; it is, however, central in disease states
IL-2	• Induces proliferation and activation of T cells, B cells, and NK cells
IL-3	• Stimulation of multilineage hematopoietic progenitors, especially when used in combination with other cytokines (SCF, IL-1, IL-6, G-CSF, GM-CSF, EPO, TPO)
IL-4 and IL-13	• Both IL-4 and IL-13 are involved in allergic reaction (induce switch to IgE)
IL-5	• Regulates production, function, survival, and migration of eosinophils • Enhances basophil number and function
IL-6	• B- and T-cell development and function • Thrombopoiesis • Acute-phase protein synthesis • Inhibition of hepatic albumin excretion • Osteoclastic bone resorption • Neural differentiation
IL-7	• Critical for T- and B-cell development
IL-8	• Potent chemoattractant agent for a variety of leukocytes, especially neutrophils • Suppresses colony formation of immature myeloid progenitors • Increases keratinocyte and endothelial cell proliferation • Increases adhesiveness of melanoma cells
IL-9	• Supports clonogenic maturation of erythroid progenitors • Acts as a mast cell differentiation factor • Protects lymphomas from apoptosis • Cooperates with IL-4 in B-cell responses • Enhances neuronal differentiation
IL-10	• Inhibits cytokine synthesis by Th1 cells and monocytes/macrophages • Stimulates B cell proliferation • Involved in transformation of B cells by Epstein-Barr virus and tumor necrosis factor (TNF) receptors
IL-11	• Best known as a thrombopoietic factor • Stimulates multilineage progenitors, erythropoiesis, myelopoiesis, and lymphopoiesis • Decreases mucositis in animal models • Stimulates osteoclast development • Inhibits adipogenesis • Stimulates proliferation of neuronal cells

Continued

Table 57-3 Major Biologic Activities of Growth Factors and Cytokines—cont'd

Biologic Activities—cont'd	
IL-12	• Proinflammatory cytokine important in resistance to infections • Th1 development • Stimulatory and inhibitory effects on hematopoiesis
IL-15	• Triggers proliferation and immunoglobulin production in preactivated B cells • Number of $CD8^+$ memory T cells may be controlled by balance of IL-15 (stimulatory) and IL-12 (inhibitory) • Stimulates proliferation of NK cells and activated $CD4^+$ or $CD8^+$ T cells • Facilitates the induction of LAK cells and CTLs • Stimulates mast cell proliferation • Promotes proliferation of hairy-cell leukemia and chronic lymphocytic leukemia cells
IL-16	• Chemoattractant for $CD4^+$ cells (T cells, monocytes, eosinophils) • May be involved in asthma and in granulomatous inflammation • Has antiviral effects on HIV-1
IL-17	• May mediate, in part, T-cell contribution to inflammation • Stimulates epithelial, endothelial, fibroblastic, and macrophage cells to express a variety of inflammatory cytokines • Promotes the capacity of fibroblasts to sustain hematopoietic progenitor growth • Promotes differentiation of dendritic cell progenitors • May be involved in the pathogenesis of rheumatoid arthritis and graft rejection
IL-18	• Promotes production of IFN-γ, TNF • Targets are T cells, NK cells, and macrophages • Promotes Th1 responses to virus
IL-19	• Induces IL-6 and TNF-α
IL-20	• Induction of genes involved in inflammation such as TNF-α, MRP14, and MCP-1
IL-21	• Mainly regulates T-cell proliferation and differentiation • Regulates cell-mediated immunity and the clearance of tumors
IL-22	• Upregulates the production of acute-phase reactants • Induces the production of ROS in resting B cells
IL-23	• A unique function of IL-23 is the preferential induction of proliferation of the memory subset of T cells
IL-24	• Induces IL-6, TNF-α, IL-1b, IL-12 and GM-CSF • Functionally it has the opposite effects with IL-10 • Infection with Ad-IL24 results in down-regulation of Bcl-2 and Bcl-XL (anti-apoptotic proteins) and upregulation of Bax and Bak (pro-apoptotic proteins) in cancer cells
IL-25	• IL-25 induces IL-4, IL-5, and IL-13 gene expression and protein production
IL-26	• Immune-protective role against viral infection
IL-27	• Early Th1 initiation • Synergizes with IL-12 in inducing IFN-γ production by T cells and NK cells
IL-28A, 28B, and 29	• Antiviral activities
IL-31	• Responsible for promoting the dermatitis and epithelial responses that characterize allergic and nonallergic diseases
IL-32	• Induces other proinflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and IL-8 • Induces IκB degradation • Phosphorylates p38 MAPK signaling pathway
IL-33	• Activates NF-κB and MAP kinases • Drives production of Th2-associated cytokines from in vitro polarized Th2 cells • Induces the expression of IL-4, IL-5, and IL-13 • Leads to severe pathologic changes in mucosal organs
IL-35	• Contributes Treg suppressor activity • Induces IL-10 and IFN-γ serum levels • Reduces induction of IL-17
IL-36	• Activates NFκB and MAP kinases • Play important role in skin biology • Involved in the initiation and regulation of immune responses
IL-37	• Regulates inflammatory responses
IL-38	• Reduces IL-36γ induced IL-8 production

AIDS, Acquired immunodeficiency syndrome; *BFU-E*, burst-forming unit-erythroid; *CTL*, cytotoxic T lymphocyte; *G-CSF*, granulocyte colony-stimulating factor; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *HIV*, human immunodeficiency virus; *IFN*, interferon; *IL*, interleukin; *LAK*, lymphokine-activated killer; *M-CSF*, macrophage colony-stimulating factor; *NK*, natural killer; *ROS*, reactive oxygen species; *SCF*, stem-cell factor; *TNF*, tumor necrosis factor; *TPO*, thrombopoietin.

multilineage mobilization of peripheral blood progenitor cells. The kinetics of progenitor release differs from that after G-CSF. Following G-CSF, peripheral blood progenitors increase almost immediately, peak at day 5 to 6, and decrease with G-CSF cessation. In contrast, PEG-MGDF resulted in a late and sustained increase in progenitors, with levels first detected on day 8 and climbing on day 12, despite cytokine discontinuation.[26] PEG-rHuMGDF has also been given to healthy subjects in a single dose of 3 mg/kg of body weight. Administration of this molecule increased the yield of platelets by a factor of nearly 4 and was associated with a quadrupling of platelet counts in the recipients of the apheresed platelets.[25]

Interleukin-1

Interleukin-1 (IL-1α and IL-1β) is the prototypic multifunctional cytokine with numerous roles in both physiological and pathological states (see Table 57-3[27-32]). This molecule influences nearly every organ system. Because IL-1 is a highly inflammatory cytokine, the margin between salutary effects and serious toxicity is exceedingly narrow.

High levels of IL-1 are seen in patients with infections (viral, bacterial, fungal, and parasitic), intravascular coagulation, and cancer (both solid tumors and hematologic malignancies). IL-1RA, a naturally occurring receptor antagonist, may also be dysregulated in inflammatory and neoplastic disease. Ultimately, it is the balance between agonist and antagonist that is probably important in determining disease manifestation.

The tumor microenvironment consists of tumor, immune, stromal, and inflammatory cells, which produce cytokines, growth factors, and adhesion molecules that promote tumor progression and metastasis. IL-1, as a pleiotropic cytokine, is known to be upregulated in many tumor types and has been implicated as a factor in tumor progression via the expression of genes associated with metastatic and angiogenic functions and growth factors. Solid tumors in which IL-1 has been shown to be upregulated include breast, colon, lung, head and neck cancers, and melanomas. Patients with IL-1–producing tumors have generally poor prognoses.[33-36]

Therefore the role of IL-1RA, as a potential novel therapeutic in cancer treatment, is being actively investigated. It currently is an approved treatment for patients with rheumatoid arthritis. This naturally occurring protein has been shown to decrease tumor growth, angiogenesis, and metastases in mouse models. There are other agents that are capable of inhibiting the inflammatory and tumor promoting effects of IL-1 such as anti-IL-1 monoclonal antibodies, the soluble IL-1R type II, and IL-1β–converting enzyme inhibitors. They are currently being used for the treatment of rheumatoid arthritis, but additional studies are necessary to determine their applicability as a novel therapy in cancer treatment.

IL-1: Clinical Trials/Applications

IL-1α and IL-1β have also been administered in clinical trials, mainly involving cancer patients.[27] In general, the acute toxicities of either isoform of IL-1 were greater after intravenous injection than after subcutaneous injection. Subcutaneous injection was associated with significant local pain, erythema, and swelling. Dose-related chills and fever were observed in nearly all patients, and even a 1-ng/kg dose was pyrogenic. Nearly all patients receiving intravenous IL-1 at doses of 100 ng/kg or greater experienced significant hypotension, probably because of induction of nitric oxide. IL-RA has been approved by the FDA for the treatment of rheumatoid arthritis.[37] Its use in metastatic disease to reduce IL-1 activity, particularly IL-1β, in cancer is being actively discussed. The most compelling design could be to add an IL-1 blocking approach to anti-VEGF or antibodies to VEGF receptors in order to reduce toxic side effects by increasing the anti-angiogenic efficacy.

Interleukin-2

IL-2 was discovered more than 30 years ago and acts as a T-cell growth and activation factor. To a lesser extent, B cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells also respond to this cytokine (see Table 57-3[38]). Following binding of IL-2 with the trimeric receptor complex, internalization occurs and cell-cycle progression is induced in association with the expression of a defined series of genes. A second functional response occurs through the IL-2βγ dimeric receptor, also known as the intermediate affinity dimeric complex (10^{-9} kDa), and involves the differentiation of several subclasses of lymphocytes into LAK cells.[39] This response occurs in patients with cancer who receive IL-2[40,41] and was originally considered to be a critical part of the anticancer effect of IL-2. LAK cells recognize and kill tumor cells, regardless of the histocompatibility expression status of fresh human tumor cells tested.[42] The multiple biologic effects of IL-2 on immune cells include the induced proliferation of antigen-stimulated T cells and induction of cytotoxicity in major histocompatibility complex (MHC)-restricted, antigen-specific T-lymphocytes, NK cells leading to non–MHC-restricted LAK cell activity, and activation of

tumoricidal monocytes. It is not clear what roles any of these effector systems have in vivo.[43]

IL-2: Clinical Trials/Applications

Because of its potent ability to stimulate cytotoxic T cells and NK cells, IL-2 has been an attractive candidate for immunotherapy of metastatic cancer, such as melanoma and kidney cancer, although with relatively low response rates and at the cost of considerable toxicity. For instance, overall response rates of renal cell cancer to IL-2 are in the range of 15% to 25%, with a complete remission rate of 5% to 10%. Complete response rates and response duration seem to favor high-dose rather than low-dose regimens. In melanoma biochemotherapy, regimens combining IL-2 and interferon-α with, for instance, cisplatin, vinblastine, and dacarbazine produce response rates of up to 60%, but this has yet to be translated into a confirmed survival effect.[38] IL-2 has also been given to leukemic patients in a variety of doses and schedules, with hints that it might be useful in remission maintenance.[38] Development of second-generation IL-2 analogs that do not induce the same high levels of secondary cytokines provides promise for further reduction of toxicities, providing that the efficacy is not dependent on these secondary effects.[44] Another approach to therapy has been to use IL-2 attached to a toxin to target and kill cancer cells bearing the IL-2 receptor. DAB389IL-2 is an IL-2 receptor (IL-2R)-specific fusion protein. It contains the enzymatic and translocation domains of the diphtheria toxin fused to human IL-2. This chimera is able to direct the cytocidal action of the diphtheria toxin enzymatic region only to cells that bear the IL-2R. DAB389IL-2 has been approved for the treatment of cutaneous T-cell lymphomas that are CD25 (IL-2 receptor) positive. Antitumor effects may also be seen in patients with other lymphoid diseases bearing the IL-2 receptor.[1]

Recent advances for the use of low-dose IL-2 therapy is promising on chronic graft-versus-host disease (GVHD), which develops in some patients who have undergone allogeneic hematopoietic stem-cell transplantation for the treatment of lymphomas and leukemias. Chronic GVHD, a systemic inflammatory disorder with pleomorphic autoimmune features, is associated with considerable morbidity and mortality. In this setting, it has been shown that IL-2 promotes both effector and regulatory T-cell responses without impairing other immune functions in the patients.[45]

About half of patients showed clinical improvement, even including an improvement in GVHD manifestations in some. These studies may provide a path for the effective use of IL-2 as a regulatory component of immunotherapy. However, the number of subjects in these trials needs to be expanded, and combinations of IL-2 with other directed immunotherapies, such as the infusion of ex vivo expanded Treg cells, could be considered as alternative approaches. However, the results of low-dose IL-2 regimens as an immunotherapy approach in cancer have been disappointing, presumably because of the combined effects of the expansion of the $CD25^+$ Treg cell population and the poor stimulation of $CD25^-$ antitumor T cells. In contrast, as a single agent or in combination with tumor vaccines, the use of high-dose IL-2 in patients with metastatic melanoma or metastatic renal cell carcinoma has led to significant therapeutic responses in up to 20% of cases and to long-term survival beyond 10 years in approximately 10% of cases. In a recent randomized, multicentral Phase III trial in advanced melanoma, the response rate was higher and progression-free survival longer with vaccine [gp100:209-217(210M) peptide vaccine] and interleukin-2 than with interleukin-2 alone.[46]

Interleukin-3

IL-3 stimulates multilineage hematopoietic progenitors (see Table 57-3[47-50]). In vitro data from supernatants of long-term bone marrow cultures suggest that marrow stromal cells produce reduced levels of IL-3 in patients with aplastic anemia.[1] IL-3 has also been implicated in patients with acute lymphocytic leukemia (ALL) with a (5:14)(q31;q32) translocation.[1] In two such patients, the translocation resulted in juxtaposition of the IL-3 gene and the Ig heavy-chain gene, and excess IL-3 transcripts were produced by the leukemic cells, perhaps explaining the eosinophilia seen in these patients.

IL-3: Clinical Trials/Applications

IL-3 has been studied in clinical trials of peripheral blood stem cell mobilization, as well as for postchemotherapy and post-transplantation cytopenias and for bone marrow failure states. Most studies have shown only modest effects of IL-3 by itself; in conjunction with other growth factors, however, significant benefits have been demonstrated. For instance, in patients with bone marrow failure treated with IL-3 followed by GM-CSF (IL-3 dosages greater than 1.2 μg/kg/d), 7 (44%) of 16 patients with severe pancytopenia had multilineage responses with normalization or near-normalization of blood counts. Although prolonged therapy was necessary to achieve maximal hematopoietic recovery, responses were durable for up to 4 years after discontinuation of treatment.[49,51] Side effects of IL-3 include dose-dependent fever, rash, fatigue, diarrhea, rigor, musculoskeletal pain, chills, headache, conjunctivitis, edema, chest pain, dyspnea, decreases in platelet counts, increases in basophilic counts, marrow fibrosis, and pulmonary edema. The tolerance to IL-3 seems to be severalfold better in patients with bone marrow failure states compared with those treated after chemotherapy.[49]

Interleukin-4 and Interleukin-13

IL-4 and IL-13 are closely related.[52] They share biologic and immunoregulatory functions on B cells, monocytes, dendritic cells, and fibroblasts. The major regulatory sequences in the IL-4 and IL-13 promoters are identical, thus explaining their restricted expression pattern in activated T cells and mast cells. Furthermore, the IL-4 and IL-13 receptors are multimeric and share at least one common chain, IL-4RA. This, together with similarities in IL-4 and IL-13 signal transduction, explains the remarkable overlap of biologic properties between these two cytokines (see Table 57-3[52-54]). The inability of IL-13 to regulate T-cell differentiation due to a lack of IL-13 receptors on T lymphocytes, however, represents a major difference between these cytokines. Therefore, despite the impact redundancy of these two molecules, regulatory mechanisms are in place to guarantee their distinct functions.

IL-4: Clinical Trials/Applications

Despite the preclinical promise of IL-4, to date clinical trials of this molecule have found the molecule to be safe and nontoxic in humans but with only sporadic antitumor activity.[54]

Interleukin-5

IL-5 is a T-cell–derived cytokine involved in the pathogenesis of atopic diseases. It specifically controls the production, activation, and localization of eosinophils. Eosinophils mediate allergic and asthmatic symptoms. T cells purified from the bronchoalveolar lavage and peripheral blood of persons with asthma secrete an elevated amount of IL-5. Therefore, agents that suppress the production or activity of IL-5 would be expected to ameliorate the pathologic effects of the allergic response (see Table 57-3[55]). Interestingly, IL-5 is secreted from Reed-Sternberg cells and may therefore be the cause of eosinophilia in patients with Hodgkin disease.[1]

Interleukin-6

IL-6 exhibits functional pleiotropy and redundancy (see Table 57-3[56-59]). IL-6 is involved in the immune response, inflammation, and hematopoiesis. Indeed, before its complete characterization, this molecule was variously referred to as interferon-β2, B-cell stimulatory factor 2, human plasmacytoma growth factor, or hepatocyte stimulatory factor. The biologic effects of IL-6 include synthesis of acute-phase reactants in the liver, as well as effects on the hypothalamic-pituitary axis, bone resorption, and both the humoral and cellular arms of the immune system.[1] As a major inducer of the acute-phase response, this cytokine may play a role in the pathogenesis of sepsis. IL-6 acts as a growth factor for myeloma/plasmacytoma, keratinocytes, mesangial cells, renal cell carcinoma, and Kaposi sarcoma and hematopoietic stem cells. On the other hand, IL-6 also inhibits the growth of myeloid leukemic cell lines and certain carcinoma cell lines.

IL-6 has been implicated as a mediator of B symptoms in lymphoma.[56] Elevated serum IL-6 concentrations have also been associated with an adverse prognosis both in Hodgkin lymphoma and in non-Hodgkin lymphoma (NHL[57,58]). In diffuse large-cell lymphoma, IL-6 levels were found to be the single most important independent prognostic factor selected in multivariate analysis for predicting complete remission rate and relapse-free survival.[57] IL-6 levels may also be exploitable as a prognostic factor in numerous solid and hematopoietic cancers.

IL-6: Clinical Trials/Applications

In patients undergoing chemotherapy or autologous transplantation, IL-6 has minimal to no platelet-enhancing activity at tolerable doses. Toxicity includes fever and anemia.[1] IL-6 has also been tested as an antitumor agent in melanoma and renal cell carcinoma. Response rates have been low (less than 15%). IL-6 inhibitors have entered the clinic. An antibody against IL-6 receptor (MRA) has been approved in Japan for the treatment of Castleman disease. An antibody against IL-6 (CNTO328) is being studied in the United States, and preliminary results show encouraging activity in lymphoma and Castleman disease. CNTO-328 is also being tested in a number of Phase I/II clinical trials in transplant-refractory myeloma and castration-resistant prostate cancer.[60] Initial results suggested that declining C-reactive protein levels during treatment may reflect biologic activity.

Interleukin-7

IL-7 promotes the proliferation of B-cell progenitors in the absence of stromal cells (see Table 57-3[61-64]). It is secreted by stromal cells in the bone marrow and thymus and is irreplaceable in the development of both B and T cells.[1] High IL-7 levels are found in states of T-cell depletion and may therefore play a role in promoting T-cell expansion.[64] High

levels of IL-7 are also found in chronic lymphocytic leukemia and in Burkitt lymphoma, and transgenic mice overexpressing the IL-7 gene show dramatic changes in lymphocyte development, which can result, in some instances, in the formation of lymphoid tumors.[1] A recent Phase I study with recombinant hIL-7 administration in refractory malignancies showed that IL-7 has potent biologic activity over a dose range that is well tolerated in humans and suggests further exploration of its possible therapeutic applications.[65] It induced a marked expansion of the T-cell mass resulting in a rejuvenated T-cell profile with an increased T-cell repertoire diversity and a decreased proportion of regulatory T cells.

Interleukin-8

IL-8 is a potent, proinflammatory chemokine that induces trafficking of neutrophils across the vascular wall (chemotaxis; see Table 57-3[66,67]). This molecule belongs to a chemokine superfamily that includes neutrophil-activating peptide-2, platelet factor-4, growth-related cytokine (GRO), and interferon inducible protein-10, all of which are responsible for the directional migration of various cells.[66] Interestingly, the IL-8 receptor demonstrates strong homology to a gene encoded by human herpesvirus-8 (HHV-8; implicated in the etiology of Kaposi sarcoma[1]). IL-8 can induce tumor growth, an effect attributed to its angiogenic activity, a property that promotes vascularization. On the other hand, antitumor effects of IL-8 have also been reported. Of interest in this regard is the fact that increased levels of IL-8 have been observed in lung carcinomas and in melanomas. IL-8 may be a growth factor for pancreatic cancer and for melanoma.[66] In melanomas, IL-8 levels correlate with the growth and metastatic potential of the tumor cells, and exposure of the cells to interferon decreases IL-8 levels and cancer cell proliferation.[67] Blocking IL-8 or IL-8R has been suggested as a therapeutic strategy.[66]

Interleukin-9

Human IL-9 was originally identified as a mitogenic factor for a human megakaryoblastic leukemia. More recently, IL-9 targets were found to encompass a wide range of cells (see Table 57-3[68,69]). There is an interesting paradox between the unresponsiveness of normal T cells to IL-9 and the potent activity of this molecule on lymphoma cells. This contrast is illustrated by the observation that murine T cells acquire the ability to respond to IL-9 after a long period of in vitro culture, while they simultaneously acquire characteristics of tumor cell lines. Observations made with transgenic mice also demonstrate the oncogenic potential of dysregulated IL-9 production, because 5% to 10% of mice that overexpress this cytokine develop lymphoblastic lymphomas.[69] In line with these findings, constitutive IL-9 production by human Hodgkin lymphomas and large-cell anaplastic lymphomas has now been clearly documented.[68]

Interleukin-10

IL-10 is a pleiotropic cytokine, initially discovered as an activity produced by murine type 2 helper T cells (Th2; see Table 57-3[70-74]). It was first called cytokine synthesis inhibitory factor because of its ability to inhibit the production of certain cytokines by Th1. Of interest, IL-10 exhibits strong DNA and amino acid sequence homology to an open reading frame—BCRF1—in the Epstein-Barr virus (EBV) genome.[1] Indeed, *BCRF1* has been called viral IL-10. The protein product of *BCRF1* (viral IL-10) exhibits properties similar to those of human IL-10. The ability of EBV to transform human B cells may be, at least in part, a ramification of the ability of viral IL-10 to stimulate B-cell proliferation.

IL-10 may have a role in the development of lymphoma through several mechanisms, including its proliferation-stimulating properties on B cells and its immunosuppressive properties that impair viral control and tumor immunosurveillance. This role has been demonstrated in a severe combined immunodeficiency (SCID) mouse model for lymphomagenesis.[1] Of interest, primary B lymphoma cells from both HIV-positive and HIV-negative patients with lymphoma (NHL) secrete substantial amounts of IL-10. Indeed, several groups of investigators have evaluated the role and prognostic significance of IL-10 in patients with NHL.[73,74] A series of studies has demonstrated that IL-10 levels are elevated in lymphoma patients and that high IL-10 levels correlate with prognosis, if an assay that detects both human and viral IL-10 is used. Assays that detect only human IL-10 yield no correlation with prognosis. These studies raise additional questions about the participation of EBV in lymphomagenesis.

In general, the biologic role of IL-10 in cancer is quite complex. The presence of IL-10 in advanced metastases and the positive correlation between serum IL-10 levels and progression of disease support the negative regulatory role of IL-10 in the tumor microenvironment. Therefore, inhibition of IL-10 could be a useful therapeutic approach as a novel cancer immunotherapy. The combination of IL-10 antagonism and immunostimulatory approaches such as cancer vaccines could also be considered as an attractive

design. However, because of its various biologic functions in the tumor microenvironment, careful clinical investigation to define the administration of anti-IL-10 agents is critically important.

Interleukin-11

IL-11 was originally characterized as a thrombopoietic factor, but it is now known to be expressed and have activity in a multitude of other systems, including the intestine, testes, and central nervous system (see Table 57-3[75,76]). Clinically, this cytokine has been approved by the FDA for amelioration of chemotherapy-induced thrombocytopenia.

IL-11 acts as a synergistic factor with IL-3, GM-CSF, and SCF to stimulate proliferation of human primary leukemia cells, myeloid leukemia cell lines, megakaryoblastic cell lines, and erythroleukemic cell lines and to stimulate leukemic blast colony formation. IL-11 mRNA expression in leukemic cells and inhibition of leukemic cell growth by IL-11 antisense oligonucleotides suggest that IL-11 may function as an autocrine growth factor in leukemic cell lines. Although IL-11 stimulates the proliferation of murine plasmacytoma cells and murine hybridoma cells, the effect of IL-11 on the growth of human myeloma/plasmacytoma cells is controversial.[75]

IL-11: Clinical Trials/Applications

A multicenter, randomized, placebo-controlled clinical trial of IL-11 versus placebo showed that IL-11 reduced the need for platelet transfusion after chemotherapy. This led to its FDA approval. Edema was the most common clinical problem associated with IL-11. Some patients developed pleural effusions, shortness of breath, and/or atrial arrhythmias.[1] Lower dosages of IL-11 (10 μg/d subcutaneously) have been reported to be safe for prolonged administration and to effectively raise platelet counts in patients who had a variety of bone marrow failure states.[76] Given the high expression of IL-11Rα protein and the proposed role of IL-11/IL-11Rα signaling in bone metastasis, it has been considered as a candidate target for primary and metastatic osteosarcoma. In animal models of osteosarcoma, it has been shown that IL-11Rα within the bone microenvironment is accessible to a circulating particle displaying a mimic of the native ligand, IL-11, and strongly accumulates within the tumor. The data indicated that IL-11Rα is a candidate target in human osteosarcoma and may serve as a target for ligand-directed delivery of agents against this disease.[77] More recently, it has been also shown that the intravenous injection of IL-11Rα-CAR T cells induced the regression of established osteosarcoma lung metastases by an ex-vivo model using genetically modified T cells targeting IL-11Rα.[78]

Interleukin-12

IL-12 is an NK cell stimulatory factor and is crucial to the development of Th1 cells.[79] There seems to be a common pathway leading from the innate immune response to adaptive immunity-intracellular pathogens to stimulate macrophages to produce IL-12, which then promotes the development of Th1 cells from a naïve cell population. This pathway may be exploitable in the design of novel immunotherapies and vaccines (see Table 57-3[79-81]). IL-12 is a potent proinflammatory molecule that is essential for resistance to bacterial, fungal, and parasitic infections. It is produced within a few hours of infection, activates NK cells, and, through its ability to induce interferon (IFN)-γ production, enhances the phagocytic and bacteriocidal activity of phagocytic cells and their ability to release proinflammatory cytokines, including IL-12 itself. IL-12 is also a key immunoregulatory molecule, especially of Th1 responses. It is produced during the early phases of infection and inflammation and sets the stage for the ensuing antigen-specific immune response, favoring differentiation and function of the Th1 T cells while inhibiting the differentiation of the Th2 T cells. IL-12 also enhances the generation of cytotoxic T cells and LAK cells. IL-12 synergizes with other hematopoietic factors to promote survival and proliferation of early multipotent hematopoietic progenitor cells and lineage-committed precursor cells.[1] Although IL-12 has mostly stimulatory effects on hematopoiesis in vitro, IL-12 treatment in vivo decreases bone marrow hematopoiesis and both transient anemia and neutropenia.

IL-12: Clinical Trials/Applications

IL-12 has the potential for exploitation in the treatment of allergy and as an adjuvant for infectious disease therapy.[80] In addition, the ability of IL-12 to revert existing states of tolerance or anergy makes it a candidate for use in the composition of vaccines for infectious agents or tumors. Phase I clinical trials have begun in oncology (with an emphasis on melanomas, renal cell carcinomas, and cutaneous T-cell lymphomas) as well as in the setting of HIV infection and chronic hepatitis B and C. In early clinical testing, IL-12 has shown limited benefit in patients with almost any malignancies. Moreover, in a clinical trial

testing a combination of IL-12 and rituximab in patients with follicular B-cell non-Hodgkin lymphoma, those treated with IL-12 showed a lower response rate.[82] Further studies showed that extended exposure to IL-12 induced T-cell exhaustion and contributed to the poor prognosis in these patients.[83] Long-term exposure of freshly isolated human CD4+ T cells to IL-12 caused T-cell dysfunction and induced expression of TIM-3 (a T-cell immunoglobulin and mucin domain protein with a known role in T-cell exhaustion), in vitro. TIM-3 was required for the negative effect of IL-12 on T-cell function. Importantly, TIM-3 also was highly expressed on intratumoral T cells that displayed marked functional impairment. These findings identify IL-12– and TIM-3–mediated exhaustion of T cells as a mechanism for poor clinical outcome when IL-12 is administered to follicular B-cell non-Hodgkin lymphoma patients.

Interleukin-15

IL-15 shares biologic activities with IL-2 (see Table 57-3[84,85]). Like IL-2, IL-15 is able to trigger both proliferation of and immunoglobulin production by normal B lymphocytes. IL-15 also stimulates the proliferation of NK cells and activated CD4+ and CD8+ T cells, and it facilitates the induction of cytolytic effector cells (such as LAK cells). Finally, the numbers of CD8+ memory T cells are maintained in animals by a balance between the stimulatory effect of IL-15 and the suppressive effects of IL-12. IL-15 responsiveness distinguishes malignant B cells from normal B lymphocytes. In contrast to normal B lymphocytes, which require preactivation in order to proliferate in response to IL-15, leukemic cells from patients with chronic B-cell malignancies proliferate in response to IL-15 regardless of in vitro preactivation. Two decades after its discovery, IL-15 is now one of the most promising new candidates in cancer immunotherapy, as well as for the treatment of infectious diseases. IL-15 is considered as a strong potential cancer therapeutic because of its ability to stimulate NK and CD8+ T cells without inducing capillary leak syndrome. Moreover, it does not trigger Tregs or suppressor cells that might alter its therapeutic benefits. The Phase I study of recombinant hIL-15 in refractory metastatic malignant melanoma and metastatic renal cell cancer is an active first-in-humans study conducted by the National Cancer Institute's Center for Cancer Research.

Interleukin-16

Cruikshank and Center first described IL-16 in 1982 (see Table 57-3[86]). They found that this molecule was a lymphocyte chemoattractant factor expressed by mitogen-stimulated human peripheral blood mononuclear cells (PBMCs). IL-16 has been implicated in several conditions, including asthma and granulomatous inflammation.[86] It may also have antiviral effects in the context of HIV-1.[1]

Interleukin-17

Human IL-17 was originally identified by Rouvier and colleagues (see Table 57-3[87,88]). Of interest, this molecule has 72% overall sequence identity at the amino acid level with open reading frame 13 of herpesvirus saimiri.[1] Although limited in number, studies suggest that IL-17 may be a soluble factor by which T cells induce or contribute to inflammation,[88] IL-17 can also stimulate epithelial, endothelial, and fibroblastic cells and macrophages to express a variety of cytokines. The cytokines released after exposure to IL-17 seem to be cell specific. For instance, fibroblast cells produce IL-1, G-CSF, IFN-γ, IL-6, and IL-8 in response to IL-17, and macrophages produce tumor necrosis factor (TNF)-α, IL-1β, IL-1Rα, IL-6, IL-10, and IL-12. IL-17 also exhibits indirect hematopoietic activity by enhancing the capacity of fibroblasts (through stimulation of growth factor release) to sustain the proliferation of CD34+ hematopoietic progenitors and their differentiation into neutrophils.[88] IL-17 can also promote the maturation of dendritic cell progenitors. Because IL-17 acts to differentiate early dendritic cells, it has been implicated in host T-cell allostimulation and graft rejection.[1]

Interleukin-18

IL-18 (IFN-inducing factor) was first described as a serum activity that induced IFN-γ production in mouse spleen cells.[1] It is related to the IL-1 family of genes (see Table 57-3[29,89,90]). IL-18 has a molecular weight of 18 to 19 kDa and has homology to IL-1.[89] Like IL-1β, IL-18 is initially synthesized as an inactive precursor molecule (pro-IL-18) lacking a signal peptide and is cleaved by ICE to yield an active molecule.[1] T lymphocytes, NK cells, and macrophages are primary targets for IL-18. For example, IL-18 directly stimulates the production of TNF in human blood CD4+ T lymphocytes and NK cells and plays an important role in promoting a long-lasting Th1 lymphocyte response to viral antigens. IL-18 does not seem to be an endogenous pyrogen but may nevertheless contribute to inflammation

and fever because it is a potent inducer of TNF, chemokines, and IFN.[90] In the case of IFN-γ induction, IL-18 acts as a costimulant with mitogens or IL-2. Indeed, mice deficient in ICE, the molecule that cleaves pro-IL-18 to its mature form, fail to produce IFN-γ in response to endotoxin.

More specifically in cancer, IL-18 plays a pivotal role in inflammation and immune responses during cancer progression. In patients with various cancers, increased IL-18 serum levels accompany tumor progression and have a negative prognostic impact. In the absence of Th1-like cytokines, IL-18 alone accelerates tumor progression. Moreover, IL-18 could drive the expression of PD-1 (Programmed Death-1) on mature NK cells with immunosuppressive functions that could act as negative regulator of general NK responses.[91] Therefore, systemic depletion of IL-18 by a binding protein is vital to stimulate NK cell–dependent immunosurveillance in tumor models. Because of this definition of IL-18 as an immunosuppressive cytokine in cancer, it is important to include this information in our novel clinical implementations of anti-PD-1 antibodies in human malignancies that produce IL-18.

Interleukin-19

IL-19 is one of the members of the human IL-10 family of cytokines (see Table 57-3[92]). IL-19 shares 21% amino acid identity with IL-10, and the exon/intron structure of IL-19 is similar to that of the human IL-10 gene, comprising five exons and four introns within the coding region of the IL-19 cDNA. The expression of IL-19 mRNA can be induced in monocytes by lipopolysaccharides or GM-CSF.

Interleukin-20

IL-20 was discovered as another IL-10–related cytokine. It induces keratinocyte proliferation and causes aberrant epidermal differentiation in the skin.[93] The IL-20 receptor complex is described as a heterodimer of two orphan class II cytokine receptor subunits termed *IL-20Rα* and *IL-20Rβ* (see Table 57-3[93-95]). Recombinant IL-20 binds to its receptor on keratinocytes and stimulates a STAT3-containing signal transduction pathway.[95] Experimental evidence suggests a role for IL-20 and its receptor in psoriasis, a multigenic skin disease characterized by increased keratinocyte proliferation and differentiation. Clinical applications are currently under consideration.

Interleukin-21

IL-21, a cytokine most closely related to IL-2 and IL-15 (see Table 57-3[94,96,97]), is involved in the proliferation and maturation of NK cell populations from bone marrow, as well as in the proliferation of mature B-cell and T-cell populations.[96] IL-21 has been implicated in the activation of innate immune responses and in the Th1 response. IL-21 also plays a critical role in regulating immunoglobulin production of B cells.[97]

IL-21 is emerging as a key T-cell and NK cell immunoregulatory cytokine with multiple effects.[98-100] It is produced mainly by activated $CD4^+$ T cells, $CD4^+$ follicular helper cells, and activated NK cells. In NK cells, IL-21 is a cell growth factor that induces NK cell expansion[101] and promotes cytotoxic activity, especially through antibody-dependent cellular cytotoxicity (ADCC) in cancer.[99,102,103] IL-21 also helps drive cytotoxic $CD8^+$ T-cell activity by enhancing the expression of perforin and granzymes.[99,103] However, in T cells, the effects of IL-21 can be pleiotropic and may depend on the state of differentiation of the T cell. In naïve and memory T cells, IL-21 promotes the maintenance of a memory phenotype by facilitating the stable expression of CD28 and other memory T-cell markers associated with the preservation of robust proliferative potential of the effector-memory cells after initial antigen-specific expansion.[98,104] IL-21 has also been found to inhibit Foxp3 expression and increase the expansion of cytolytic $CD8^+$ T cells during an immune response.[105] Another mechanism of action of IL-21 that has emerged recently is telomerase. In both NK cells and T cells, IL-21 has been shown to induce or maintain telomerase expression and prevent telomere erosion during cell expansion.[101] Nevertheless, by itself, IL-21 is a relatively poor driver of T-cell division. However, combinations of IL-21 with other gamma chain cytokines, such as IL-2 and IL-15, have been found to synergize in driving T-cell division and maintenance of an effector-memory phenotype. This has been demonstrated during the activation and expansion of tumor antigen–specific T cells in melanoma, for example, where IL-21 maintained CD28 expression in the primed MART-1–specific T cells and in tumor-infiltrating lymphocytes (TIL) expanded with IL-21 in synergy with IL-15.[104,106] Another key function for IL-21 in human T cells seems to be in facilitating the expression of effector molecules in $CD8^+$ T cells. For example, IL-21 was found to restore and increase perforin expression in $CD8^+$ T cells from HIV-infected patients.[107,108] Clinical trials with IL-21 have begun recently.

IL-21: Clinical Trials/Applications

A number of small Phase I and Phase II clinical trials with recombinant IL-21 (Denenicokin from Zymogenetics) have been completed with a dose range between 1 and 300 μg/kg given either subcutaneously or intravenously.[109] A recent Phase I dose-escalation clinical trial using subcutaneous IL-21 injection (3 to 300 μg/kg) 3 days a week for 8 or 16 weeks in metastatic cancer patients (melanoma and renal cell carcinoma) found that it was relatively well tolerated with a maximum tolerated dose of 200 μg/kg.[110] Interferon-γ, perforin, and granzyme B mRNA expression in peripheral blood and granzyme B protein expression in both $CD8^+$ T cells and NK cells were found at the higher dose range, consistent with the activation of cytotoxic lymphocytes.[110] Some clinical responses were also noted. Similar results were found in a Phase I trial using intravenous IL-21 infusion.[111,112] Other clinical trials are now combining IL-21 with tumor cell targeting monoclonal antibodies, such as anti-EGFR, to facilitate ADCC by driving NK cell activation. A regimen of IL-21 with cetuximab (anti-EGFR) in colorectal cancer patients was well tolerated, with some clinical benefit noted.[113] However, in other circumstances, dose-limiting hematologic toxicities in combination with other agents, such as sunitinib in renal cell carcinoma patients, have been found.[114] Overall, IL-21 is a promising agent against cancer, and its immunological mechanism of action suggests that it should be tested in combination with targeted monoclonal antibodies to accentuate ADCC[102] or with prior cytotoxic therapy to release tumor antigens and also activate NK cells, where the cytokine can then enhance an induced NK cell or T-cell response.

Interleukin-22

IL-22 was originally described as an IL-9–inducible gene and called IL-TIF.[155] IL-22 activities include induction of the acute-phase response in hepatocytes. These activities are mediated through a heterodimeric receptor composed of the IL-22R subunit and the chain of IL-10R.[116] In addition to its cellular receptor, IL-22 binds to a secreted class II cytokine receptor family member that acts as a natural IL-22 antagonist (see Table 57-3[115,116]). IL-22 is mainly produced by activated T-cell subpopulations (Th22, Th1, Th17), and TNF-α, IL-17, and IFN-α amplify its effects on cells of the skin and gastrointestinal and respiratory systems. Blood and lesional skin samples from psoriasis patients demonstrate high levels of IL-22; it acts principally on keratinocytes and does not influence the immune system.[117] IL-22 probably plays the main role in the induction of psoriatic epidermal alterations, and therapeutic approaches that counteract IL-22 are under consideration as of today.

Interleukin-23

IL-23 is a member of the IL-6 family of cytokines and is closely related in structure to IL-12. IL-23 and IL-12 are heterodimeric cytokines that share the p40 subunit, and each has a unique second subunit, IL-23p19 and IL-12p35, respectively (see Table 57-3[118]). In addition to the close structural relationship between IL-23 and IL-12, their heterodimeric receptors share the IL-12Rb chain, and these cytokines have similar properties.[118]

Interleukin-24

When IL-24 was discovered, it was designated as melanoma differentiation–associated gene-7 (mda-7), because it was identified by subtractive hybridization after the treatment of melanoma cells with IFN-β and mezerein, which caused their terminal differentiation and growth arrest.[119] Later, it was recognized that mda-7 encodes a secreted protein that exhibits significant homology to IL-10 (see Table 57-3[94,120-124]). Then, this molecule was officially designated as IL-24.[120] Human IL-24 is secreted by activated PBMCs and is the ligand for two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2.[121] IL-24 also acts as a tumor suppressor gene, and the protein product was found to be constitutively expressed by melanocytes, nevus cells, and some primary melanomas but not metastatic lesions of melanoma.[122,123] It is an example of a tumor suppressor gene exhibiting immune stimulatory properties.[124]

IL-24: Clinical Trials/Applications

Because of the tumor suppressor characteristics of IL-24, it may potentially be used for cancer treatments in the clinic. Gene transfer of IL-24 was studied in the Phase I setting using intratumoral injections of Ad-mda7/IL-24 (INGN 241) in 28 patients with resectable solid tumors.[125] It has been reported that intratumoral administration of INGN 241 is well tolerated and induces apoptosis in a large percentage of tumor cells.[125,126] Future clinical approaches will

be required to determine whether IL-24 represents a viable therapeutic in cancer and whether it could be rationally combined with other cancer therapeutics to improve tumor killing in patients.

Interleukin-25

IL-25 was recently identified as a cytokine that is structurally related to IL-17 and induces IL-4, IL-5, and IL-13 gene expression (see Table 57-3[127]). The induction of these cytokines results in Th2-like responses marked by increased serum IgE, IgG1, and IgA concentrations, blood eosinophilia, and epithelial cell hyperplasia. Little is known about this newly discovered cytokine besides the fact that IL-25 is derived from Th2 T cells and that it can amplify allergic-type inflammatory responses by its actions on other cells.

Interleukin-26

Subtraction hybridization coupled with representational differential analysis identified IL-26/AK155 as a gene upregulated in human T cells following infection with herpesvirus saimiri (HVS[1]). It has the capacity to transform these cells in culture (see Table 57-3[94,128]). The IL-26 protein has 24.7% amino acid identity and 47% amino acid similarity with human IL-10. Structural analysis revealed that IL-26 contains six helices with four highly conserved cysteine residues, which are assumed to be relevant for dimer formation, as is the case with IL-10. It was determined that IL-26 mRNA is specifically overexpressed by T cells after HVS transformation.

Interleukin-27

In 2002, Pflanz and colleagues[129] described a new heterodimeric cytokine related to IL-12. This cytokine was designated IL-27. IL-27 acts together with IL-12 to trigger IFN-γ production by naïve $CD4^+$ T cells (see Table 57-3[129,130]). They also identified IL-27 as the ligand for TCCR/WSX-1, a novel member of the class I cytokine receptor family shown to be important for Th1 development.[1] Recent studies have found that IL-27 has the ability to induce tumor-specific antitumor activity and protective immunity and that the antitumor activity is mediated mainly through $CD8^+$ T cells and IFN-γ.[130] IL-27, similar to IL-12, shows antitumor activity in different tumors via induction of NK and CTL response or inhibition of angiogenesis by the induction of CXCL9 and CXCL10.[131,132] It has also an antiproliferative effect on melanoma cells,[133] which express its receptor (and multifunctional antitumor roles in multiple myeloma[134]).

Interleukin-28 and Interleukin-29

The IL-28 family has been identified from the human genomic sequence, and the member cytokines have been designated interleukin 28A (IL-28A), IL-28B, and IL-29. These molecules are distantly related to type I IFNs and the IL-10 family. IL-28 and IL-29 are induced by viral infection and show antiviral activity. Moreover, IL-28 and IL-29 interact with a heterodimeric class II cytokine receptor that consists of IL-10Rβ and an orphan class II receptor chain, designated IL-28Rα. This newly described cytokine family may serve as an alternative to type I IFNs in providing immunity to viral infection (see Table 57-3[135]). Their antiviral effects and gene-inducing activities have been compared with those of IFNs, but the data are very limited at present. Their clinical use has the potential for the treatment of hepatitis C virus (HCV) infection based on comparative analyses of their transcriptome and antiviral properties.[136]

Interleukin-31

IL-31 has been identified as a four-helix bundle cytokine that is preferentially produced by T-helper type 2 cells. IL-31 signals through a receptor composed of IL-31 receptor A and oncostatin M receptor. Expression of IL-31 receptor A and oncostatin M receptor mRNA was induced in activated monocytes, whereas epithelial cells expressed both mRNAs constitutively (see Table 57-3[137]). More specifically, the data indicated that IL-31 might be involved in promoting the dermatitis and the epithelial responses that characterize allergic and nonallergic diseases. It is also known that binding of IL-31 to its receptor activates JAK/STAT, PI3K/AKT, and MAPK pathways.[138]

Interleukin-32

IL-32 is a recently discovered proinflammatory cytokine that induces TNF-α, IL-1β, IL-6, and chemokines (see Table 57-3[139]). The proinflammatory activity of IL-32 seems to take place after degradation of IKβ, leading to activation of

NFκB as well as phosphorylation of mitogen-activated protein p38.[139] IL-32 was originally identified as a transcript, NK cell transcript 4 (NK4), the expression of which was increased in activated NK cells. It has been very recently demonstrated that NK4 is secreted from several cells on the stimulation of some inflammatory cytokines, such as IL-18, IL-1β, IFN-γ, and IL-12.[140] The gene encoding IL-32 resides at chromosome 16 p13.3, and six mRNA transcripts resulting from mRNA splicing are presently known, but the functional differences between these isoforms remain unknown.[141] The interaction between tumor and immune cells is responsible for overall tumor progression or regression. Cytokines can act as inducers via activation of T cells and NK cells in the tumor tissues, which results in the apoptotic cell death of tumor cells. A recent study revealed the role of IL-32 in STAT3 and NFκB pathways during cancer development, which are known to stimulate prosurvival, proliferative, anti-apoptotic, and pro-angiogenic genes in cancer development.[142] This study showed that IL-32 inhibits constitutively activated STAT3 and NFκB signaling, reduces the production of pro-inflammatory cytokines, and increased production of anti-inflammatory cytokines in colon cancer and melanoma cells. At this point, it is clear that IL-32 may play significant pathophysiological roles in cancer development.

Interleukin-33

IL-33, a member of the IL-1 family, which mediates its biologic effects via IL-1 receptor ST2, activates NFκB and MAP kinases, and it drives production of T(H)2-associated cytokines from in vitro polarized T(H)2 cells. In vivo, IL-33 induces the expression of IL-4, IL-5, and IL-13 and leads to severe pathologic changes in mucosal organs (see Table 57-3[143]). Analysis of a panel of human and mouse cDNA libraries by real-time quantitative polymerase chain reaction (PCR) showed that IL-33 mRNA is broadly expressed in many tissues. At the protein level, it is expressed by many cell types following pro-inflammatory stimulation, such as fibroblasts, epithelial cells, and endothelial cells.[144] In the absence of pro-inflammatory stimuli, IL-33 localizes to the nucleus.[145,146] Nuclear localization of full-length IL-33 is mediated by the amino terminus, which contains a nuclear localization sequence and a short chromatin-binding motif. IL-33 has a dual role in disease; it is associated with host protection against infections by promoting T(H)2 cells but can also aggravate T(H)2 and mast cells in inflammatory diseases.[144] The role of IL-33 in bacterial and viral infections remains to be explored, and its contribution to many inflammatory diseases is vital not only for inflammation but also for many cancers, which could be caused by chronic inflammation via induction of genetic and epigenetic aberrations in affected cells.

Interleukin-35

IL-35 represents a new member of the heterodimeric IL-12 cytokine family. It is a novel inhibitory cytokine that is produced by Treg cells and contributes to their suppressive activity.

In vivo studies showed that IL-35 reduces the incidence of arthritis and pathologic features of collagen-induced arthritis in mice by regulating serum levels of IL-10, IFN-γ, and IL-17.[147] Because IL-35 may be secreted exclusively by Treg cells and other cell populations with regulatory potential, it represents a novel potential target for the therapeutic manipulation of Treg activity to treat cancer and autoimmune diseases.

Interleukin-36

IL-36α, IL-36β, and IL-36γ are members of the IL-1 family of cytokines. They signal through the IL-1 receptor family members IL-1RL2 and IL-1RAcP to activate NFκB and MAPKs[148] and play an important role in skin biology. Similar to the classical IL-1 cytokines, IL-36 cytokines are also involved in the regulation of immune responses. IL-36s are expressed in a restricted manner, primarily in the skin, airway, and other epithelial tissues, whereas the receptors are more commonly expressed,[149] such as in the lung, joint, gut, kidney, and brain.

Interleukin-37

Since the discovery of IL-1 in 1977, the nomenclature has been continuously evolving, and there have been proposals for the assignment of new members to the IL-1 family.[150] IL-37 was originally defined as IL-1 family member 7 (IL-1F7), and transcripts are detected in lymph nodes, thymus, bone marrow, lung, testis, uterus, and placenta.[149] The anti-inflammatory features of IL-37 have been demonstrated in vitro. TGF-β and several Toll-like receptor (TLR) ligands induce production of high levels of IL-37 by PBMCs; proinflammatory cytokines such as IL-18, IFN-γ, IL-1β, and TNF moderately increase IL-37 levels.[151] In addition, expression of IL-37 in monocytic cells has been shown to reduce several intracellular kinases important for transducing proinflammatory

signals, such as focal adhesion kinase (FAK), STAT1, p38 MAPK, and c-jun. Therefore IL-37 is considered as one of the many key modulators of inflammation.

Interleukin-38

IL-38, originally named IL-1 family member 10, is one of 11 members of the family. Earlier studies show that IL-38 polymorphisms are associated with psoriatic arthritis and ankylosing spondylitis and suggested a role in the pathogenesis of these inflammatory diseases.[152,153] It has recently been shown that IL-38 binds to the IL-36R, as does IL-36Ra, and that IL-38 and IL-36Ra have similar biologic effects on immune cells and reduce the production of IL-17 and IL-22, which is similar to that caused by the IL-36 receptor antagonist in the same PBMC cultures.[154]

Conclusion

Many, if not most, cytokines and their respective natural inhibitors are ubiquitously expressed and have myriad biologic properties that influence virtually every organ system. It is already apparent that these molecules may also be effective in allergic, inflammatory, and autoimmune diseases, as well as in cancer therapeutics. The emerging understanding of their role and the availability of recombinant molecules for clinical therapeutics suggest that their application is still evolving and will ultimately affect many areas of medicine.

Key References

1. Ekmekcioglu S, Kurzrock R, Grimm EA. Cytokines and hematopoietic growth factors. In: Hong WK, ed. *Cancer Medicine*. Shelton, Conn: People's Medical Publishing House-USA/BC Decker; 2010:686-709.
2. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem*. 2002;277:29355-29358.
3. *Online Mendelian Inheritance in Man*. Gene map. National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/Omim/getmap.cgi. Accessed June 17, 2005.
5. Bennett CL, Silver SM, Djulbegovic B, et al. Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia. *JAMA*. 2008;299:914-924.
6. Bohlius J, Schmidlin K, Brillant C, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials. *Lancet*. 2009;373:1532-1542.
7. Rizzo JD, Brouwers M, Hurley P, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood*. 2010;116:4045-4059.
8. Pronzato P, Cortesi E, van der Rijt CC, et al. Epoetin alfa improves anemia and anemia-related, patient-reported outcomes in patients with breast cancer receiving myelotoxic chemotherapy: results of a European, multicenter, randomized, controlled trial. *Oncologist*. 2010;15:935-943.
11. Crawford J. Clinical uses of pegylated pharmaceuticals in oncology. *Cancer Treat Rev*. 2002;28(suppl A):7-11.
12. Smith TJ, Khatcheressian J, Lyman GH, et al. 2006 update of recommendations for the use of white blood cell growth factors: an evidence-based clinical practice guideline. *J Clin Oncol*. 2006;24:3187-3205.
13. Kurzrock R. Thrombopoietic factors in chronic bone marrow failure states: the platelet problem revisited. *Clin Cancer Res*. 2005;11:1361-1367.
19. Metcalf D. Thrombopoietin—at last. *Nature*. 1994;369:519-520.
33. Apte RN, Krelin Y, Song X, et al. Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour-host interactions. *Eur J Cancer*. 2006;42:751-759.
35. Elaraj DM, Weinreich DM, Varghese S, et al. The role of interleukin 1 in growth and metastasis of human cancer xenografts. *Clin Cancer Res*. 2006;12:1088-1096.
36. Qin Y, Ekmekcioglu S, Liu P, et al. Constitutive aberrant endogenous interleukin-1 facilitates inflammation and growth in human melanoma. *Mol Cancer Res*. 2011;9:1537-1550.
37. Cohen SB. The use of anakinra, an interleukin-1 receptor antagonist, in the treatment of rheumatoid arthritis. *Rheum Dis Clin North Am*. 2004;30:365-380, vii.
38. Atkins MB. Interleukin-2: clinical applications. *Semin Oncol*. 2002;29(3 Suppl 7):12-17.
42. Grimm EA, Mazumder A, Zhang HZ, et al. Lymphokine-activated killer cell phenomenon. Lysis of natural killer–resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med*. 1982;155:1823-1841.
44. Heaton KM, Ju G, Grimm EA. Human interleukin 2 analogues that preferentially bind the intermediate-affinity interleukin 2 receptor lead to reduced secondary cytokine secretion: implications for the use of these interleukin 2 analogues in cancer immunotherapy. *Cancer Res*. 1993;53:2597-2602.
45. Koreth J, Matsuoka K, Kim HT, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. 2011;365:2055-2066.
46. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364:2119-2127.
51. Wu HH, Talpaz M, Champlin RE, et al. Sequential interleukin 3 and granulocyte-macrophage-colony stimulating factor therapy in patients with bone marrow failure with long-term follow-up of responses. *Cancer*. 2003;98:2410-2419.
60. Dorff TB, Goldman B, Pinski JK, et al. Clinical and correlative results of SWOG S0354: a phase II trial of CNTO328 (siltuximab), a monoclonal antibody against interleukin-6, in chemotherapy-pretreated patients with castration-resistant prostate cancer. *Clin Cancer Res*. 2010;16:3028-3034.
65. Sportes C, Babb RR, Krumlauf MC, et al. Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. *Clin Cancer Res*. 2010;16:727-735.
72. Dumoutier L, Renauld JC. Viral and cellular interleukin-10 (IL-10)-related cytokines: from structures to functions. *Eur Cytokine Netw*. 2002;13:5-15.
77. Lewis VO, Ozawa MG, Deavers MT, et al. The interleukin-11 receptor alpha as a candidate ligand-directed target in osteosarcoma: consistent data from cell lines, orthotopic models, and human tumor samples. *Cancer Res*. 2009;69:1995-1999.
78. Huang G, Yu L, Cooper LJ, et al. Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases. *Cancer Res*. 2012;72:271-281.
82. Ansell SM, Geyer SM, Maurer MJ, et al. Randomized phase II study of interleukin-12 in combination with rituximab in previously treated non-Hodgkin's lymphoma patients. *Clin Cancer Res*. 2006;12(20 Pt 1):6056-6063.
83. Yang ZZ, Grote DM, Ziesmer SC, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Invest*. 2012;122:1271-1282.

91. Terme M, Ullrich E, Aymeric L, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res*. 2011;71:5393-5399.
94. Pestka S, Krause CD, Sarkar D, et al. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol*. 2004;22:929-979.
98. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol*. 2008;26:57-79.
99. Andorsky DJ, Timmerman JM. Interleukin-21: biology and application to cancer therapy. *Expert Opin Biol Ther*. 2008;8:1295-1307.
100. Sarra M, Franze E, Pallone F, et al. Targeting interleukin-21 in inflammatory diseases. *Expert Opin Ther Targets*. 2011;15:695-702.
101. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One*. 2012;7:e30264.
102. Watanabe M, Kono K, Kawaguchi Y, et al. Interleukin-21 can efficiently restore impaired antibody-dependent cell-mediated cytotoxicity in patients with oesophageal squamous cell carcinoma. *Br J Cancer*. 2010;102:520-529.
103. Smyth MJ, Teng MW, Sharkey J, et al. Interleukin 21 enhances antibody-mediated tumor rejection. *Cancer Res*. 2008;68:3019-3025.
104. Li Y, Bleakley M, Yee C. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. *J Immunol*. 2005;175:2261-2269.
105. Li Y, Yee C. IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific $CD8^+$ cytotoxic T lymphocytes. *Blood*. 2008;111:229-235.
106. Li Y, Liu S, Hernandez J, et al. MART-1-specific melanoma tumor-infiltrating lymphocytes maintaining CD28 expression have improved survival and expansion capability following antigenic restimulation in vitro. *J Immunol*. 2010;184:452-465.
107. Parmigiani A, Pallin MF, Schmidtmayerova H, et al. Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells. *Hum Immunol*. 2011;72:115-123.
108. Strbo N, de Armas L, Liu H, et al. IL-21 augments natural killer effector functions in chronically HIV-infected individuals. *AIDS*. 2008;22:1551-1560.
109. Hashmi MH, Van Veldhuizen PJ. Interleukin-21: updated review of Phase I and II clinical trials in metastatic renal cell carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. *Expert Opin Biol Ther*. 2010;10:807-817.
110. Schmidt H, Brown J, Mouritzen U, et al. Safety and clinical effect of subcutaneous human interleukin-21 in patients with metastatic melanoma or renal cell carcinoma: a phase I trial. *Clin Cancer Res*. 2010;16:5312-5319.
111. Davis ID, Skrumsager BK, Cebon J, et al. An open-label, two-arm, phase I trial of recombinant human interleukin-21 in patients with metastatic melanoma. *Clin Cancer Res*. 2007;13:3630-3636.
112. Thompson JA, Curti BD, Redman BG, et al. Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. *J Clin Oncol*. 2008;26:2034-2039.
113. Steele N, Anthony A, Saunders M, et al. A phase 1 trial of recombinant human IL-21 in combination with cetuximab in patients with metastatic colorectal cancer. *Br J Cancer*. 2012;106:793-798.
114. Grunwald V, Desar IM, Haanen J, et al. A phase I study of recombinant human interleukin-21 (rIL-21) in combination with sunitinib in patients with metastatic renal cell carcinoma (RCC). *Acta Oncol*. 2011;50:121-126.
117. Sabat R, Wolk K. Research in practice: IL-22 and IL-20: significance for epithelial homeostasis and psoriasis pathogenesis. *J Dtsch Dermatol Ges*. 2011;9:518-523.
124. Chada S, Mhashilkar AM, Ramesh R, et al. Bystander activity of Ad-mda7: human MDA-7 protein kills melanoma cells via an IL-20 receptor-dependent but STAT3-independent mechanism. *Mol Ther*. 2004;10:1085-1095.
125. Cunningham CC, Chada S, Merritt JA, et al. Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol Ther*. 2005;11:149-159.
126. Tong AW, Nemunaitis J, Su D, et al. Intratumoral injection of INGN 241, a nonreplicating adenovector expressing the melanoma-differentiation associated gene-7 (mda-7/IL24): biologic outcome in advanced cancer patients. *Mol Ther*. 2005;11:160-172.
128. Sheikh F, Baurin VV, Lewis-Antes A, et al. Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. *J Immunol*. 2004;172:2006-2010.
130. Hisada M, Kamiya S, Fujita K, et al. Potent antitumor activity of interleukin-27. *Cancer Res*. 2004;64:1152-1156.
131. Shimizu M, Shimamura M, Owaki T, et al. Antiangiogenic and antitumor activities of IL-27. *J Immunol*. 2006;176:7317-7324.
132. Airoldi I, Cocco C, Giuliani N, et al. Constitutive expression of IL-12R beta 2 on human multiple myeloma cells delineates a novel therapeutic target. *Blood*. 2008;112:750-759.
133. Yoshimoto T, Morishima N, Mizoguchi I, et al. Antiproliferative activity of IL-27 on melanoma. *J Immunol*. 2008;180:6527-6535.
134. Cocco C, Giuliani N, Di Carlo E, et al. Interleukin-27 acts as multifunctional antitumor agent in multiple myeloma. *Clin Cancer Res*. 2010;16:4188-4197.
135. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol*. 2003;4:63-68.
136. Diegelmann J, Beigel F, Zitzmann K, et al. Comparative analysis of the lambda-interferons IL-28A and IL-29 regarding their transcriptome and their antiviral properties against hepatitis C virus. *PLoS One*. 2010;5:e15200.
137. Dillon SR, Sprecher C, Hammond A, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol*. 2004;5:752-760.
138. Dreuw A, Radtke S, Pflanz S, et al. Characterization of the signaling capacities of the novel gp130-like cytokine receptor. *J Biol Chem*. 2004;279:36112-36120.
139. Kim SH, Han SY, Azam T, et al. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity*. 2005;22:131-142.
140. Novick D, Rubinstein M, Azam T, et al. Proteinase 3 is an IL-32 binding protein. *Proc Natl Acad Sci U S A*. 2006;103:3316-3321.
141. Nold-Petry CA, Nold MF, Zepp JA, et al. IL-32-dependent effects of IL-1beta on endothelial cell functions. *Proc Natl Acad Sci U S A*. 2009;106:3883-3888.
142. Oh JH, Cho MC, Kim JH, et al. IL-32gamma inhibits cancer cell growth through inactivation of NF-kappaB and STAT3 signals. *Oncogene*. 2011;30:3345-3359.
143. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23:479-490.
144. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*. 2010;10:103-110.
145. Carriere V, Roussel L, Ortega N, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A*. 2007;104:282-287.
146. Baekkevold ES, Roussigne M, Yamanaka T, et al. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol*. 2003;163:69-79.
147. Niedbala W, Wei XQ, Cai B, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*. 2007;37:3021-3029.
148. Towne JE, Renshaw BR, Douangpanya J, et al. Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity. *J Biol Chem*. 2011;286:42594-42602.
150. Dinarello C, Arend W, Sims J, et al. IL-1 family nomenclature. *Nat Immunol*. 2010;11:973.
151. Nold MF, Nold-Petry CA, Zepp JA, et al. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol*. 2010;11:1014-1022.
152. Rahman P, Sun S, Peddle L, et al. Association between the interleukin-1 family gene cluster and psoriatic arthritis. *Arthritis Rheum*. 2006;54:2321-2325.
153. Chou CT, Timms AE, Wei JC, et al. Replication of association of IL1 gene complex members with ankylosing spondylitis in Taiwanese Chinese. *Ann Rheum Dis*. 2006;65:1106-1109.
154. van de Veerdonk FL, Stoeckman AK, Wu G, et al. IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proc Natl Acad Sci U S A*. 2012;109:3001-3005.

References

1. Ekmekcioglu S, Kurzrock R, Grimm EA. Cytokines and hematopoietic growth factors. In: Hong WK, ed. *Cancer Medicine*. Shelton, Conn: People's Medical Publishing House-USA/BC Decker; 2010:686-709.
2. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem*. 2002;277:29355-29358.
3. *Online Mendelian Inheritance in Man*. Gene map. National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov/Omim/getmap.cgi. Accessed June 17, 2005.
4. Rizzo JD, Lichtin AE, Woolf SH, et al. Use of epoetin in patients with cancer: evidence-based clinical practice guidelines of the American Society of Clinical Oncology and the American Society of Hematology. *J Clin Oncol*. 2002;20:4083-4107.
5. Bennett CL, Silver SM, Djulbegovic B, et al. Venous thromboembolism and mortality associated with recombinant erythropoietin and darbepoetin administration for the treatment of cancer-associated anemia. *JAMA*. 2008;299:914-924.
6. Bohlius J, Schmidlin K, Brillant C, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials. *Lancet*. 2009;373:1532-1542.
7. Rizzo JD, Brouwers M, Hurley P, et al. American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update on the use of epoetin and darbepoetin in adult patients with cancer. *Blood*. 2010;116:4045-4059.
8. Pronzato P, Cortesi E, van der Rijt CC, et al. Epoetin alfa improves anemia and anemia-related, patient-reported outcomes in patients with breast cancer receiving myelotoxic chemotherapy: results of a European, multicenter, randomized, controlled trial. *Oncologist*. 2010;15:935-943.
9. Gasson JC. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood*. 1991;77:1131-1145.
10. Wetzler M, Estrov Z, Talpaz M, et al. Granulocyte-macrophage colony-stimulating factor as a cause of paraneoplastic leukaemoid reaction in advanced transitional cell carcinoma. *J Intern Med*. 1993;234:417-420.
11. Crawford J. Clinical uses of pegylated pharmaceuticals in oncology. *Cancer Treat Rev*. 2002;28(suppl A):7-11.
12. Smith TJ, Khatcheressian J, Lyman GH, et al. 2006 update of recommendations for the use of white blood cell growth factors: an evidence-based clinical practice guideline. *J Clin Oncol*. 2006;24:3187-3205.
13. Kurzrock R. Thrombopoietic factors in chronic bone marrow failure states: the platelet problem revisited. *Clin Cancer Res*. 2005;11:1361-1367.
14. Motoyoshi K. Biological activities and clinical application of M-CSF. *Int J Hematol*. 1998;67:109-122.
15. Williams DE, Eisenman J, Baird A, et al. Identification of a ligand for the c-kit proto-oncogene. *Cell*. 1990;63:167-174.
16. Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med*. 2002;347:472-480.
17. Tricot G, Jagannath S, Desikan KR, et al. Superior mobilization of peripheral blood progenitor cells (PBPC) with r-metHuSCF (SCF) and r-metHuG-CSF (Filgrastim) in heavily pretreated multiple myeloma (MM) patients. *Blood*. 1996;88:388a-393a (abstr. suppl 1).
18. Kurzrock R, Gratwohl A, Paquette R, et al. Trilineage responses seen with stem cell factor (STEMGEN, SCF) and filgrastim (G-CSF) treatment in aplastic anemia (AA) patients (Pts). *Br J Haematol*. 1998;102:1.
19. Metcalf D. Thrombopoietin—at last. *Nature*. 1994;369:519-520.
20. Kaushansky K, Drachman JG. The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. *Oncogene*. 2002;21:3359-3367.
21. Vadhan-Raj S, Murray LJ, Bueso-Ramos C, et al. Stimulation of megakaryocyte and platelet production by a single dose of recombinant human thrombopoietin in patients with cancer. *Ann Intern Med*. 1997;126:673-681.
22. Fanucchi M, Glaspy J, Crawford J, et al. Effects of polyethylene glycol-conjugated recombinant human megakaryocyte growth and development factor on platelet counts after chemotherapy for lung cancer. *N Engl J Med*. 1997;336:404-409.
23. Glaspy J, Vredenburgh J, Demetri GD, et al. Effects of PEGylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) before high dose chemotherapy (HDC) with peripheral blood progenitor cell (PBPC) support. *Blood*. 1997;90:580a.
24. Nash R, Kurzrock R, DiPersio J, et al. Safety and activity of recombinant human thrombopoietin (rhTPO) in patients (pts) with delayed platelet recovery (DPR). *Blood*. 1997;90:262a.
25. Kuter D, McCullough J, Romo J, et al. Treatment of platelet (PLT) donors with pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) increases circulating PLT counts (CTS) and PLT apheresis yields and increases platelet increments in recipients of PLT transfusions. *Blood*. 1997;90:579a.
26. Kuter DJ, Begley CG. Recombinant human thrombopoietin: basic biology and evaluation of clinical studies. *Blood*. 2002;100:3457-3469.
27. Dinarello CA. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. *Int Rev Immunol*. 1998;16:457-499.
28. Veltri S, Smith 2nd JW. Interleukin 1 trials in cancer patients: a review of the toxicity, antitumor and hematopoietic effects. *Stem Cells*. 1996;14:164-176.
29. Sims JE. IL-1 and IL-18 receptors, and their extended family. *Curr Opin Immunol*. 2002;14:117-122.
30. Wetzler M, Kurzrock R, Estrov Z, et al. Altered levels of interleukin-1 beta and interleukin-1 receptor antagonist in chronic myelogenous leukemia: clinical and prognostic correlates. *Blood*. 1994;84:3142-3147.
31. Estrov Z, Kurzrock R, Estey E, et al. Inhibition of acute myelogenous leukemia blast proliferation by interleukin-1 (IL-1) receptor antagonist and soluble IL-1 receptors. *Blood*. 1992;79:1938-1945.
32. Estrov Z, Kurzrock R, Wetzler M, et al. Suppression of chronic myelogenous leukemia colony growth by interleukin-1 (IL-1) receptor antagonist and soluble IL-1 receptors: a novel application for inhibitors of IL-1 activity. *Blood*. 1991;78:1476-1484.
33. Apte RN, Krelin Y, Song X, et al. Effects of micro-environment- and malignant cell-derived interleukin-1 in carcinogenesis, tumour invasiveness and tumour-host interactions. *Eur J Cancer*. 2006;42:751-759.
34. Gemma A, Takenaka K, Hosoya Y, et al. Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line. *Eur J Cancer*. 2001;37:1554-1561.
35. Elaraj DM, Weinreich DM, Varghese S, et al. The role of interleukin 1 in growth and metastasis of human cancer xenografts. *Clin Cancer Res*. 2006;12:1088-1096.
36. Qin Y, Ekmekcioglu S, Liu P, et al. Constitutive aberrant endogenous interleukin-1 facilitates inflammation and growth in human melanoma. *Mol Cancer Res*. 2011;9:1537-1550.
37. Cohen SB. The use of anakinra, an interleukin-1 receptor antagonist, in the treatment of rheumatoid arthritis. *Rheum Dis Clin North Am*. 2004;30:365-380 vii.
38. Atkins MB. Interleukin-2: clinical applications. *Semin Oncol*. 2002;29(3 suppl 7):12-17.
39. Grimm EA, Owen-Schaub LB, Loudon WG, et al. Lymphokine-activated killer cells. Induction and function. *Ann N Y Acad Sci*. 1988;532:380-386.
40. Yang SC, Grimm EA, Parkinson DR, et al. Clinical and immunomodulatory effects of combination immunotherapy with low-dose interleukin 2 and tumor necrosis factor alpha in patients with advanced non-small cell lung cancer: a phase I trial. *Cancer Res*. 1991;51:3669-3676.
41. Yang SC, Owen-Schaub L, Grimm EA, et al. Induction of lymphokine-activated killer cytotoxicity with interleukin-2 and tumor necrosis factor-alpha against primary lung cancer targets. *Cancer Immunol Immunother*. 1989;29:193-198.
42. Grimm EA, Mazumder A, Zhang HZ, et al. Lymphokine-activated killer cell phenomenon. Lysis of natural killer–resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med*. 1982;155:1823-1841.
43. Owen-Schaub LB, de Mars M, Murphy Jr EC, et al. IL-2 dose regulates TNF-alpha mRNA transcription and protein secretion in human peripheral blood lymphocytes. *Cell Immunol*. 1991;132:193-200.
44. Heaton KM, Ju G, Grimm EA. Human interleukin 2 analogues that preferentially bind the intermediate-affinity interleukin 2 receptor lead to reduced secondary cytokine secretion: implications for the use of these interleukin 2 analogues in cancer immunotherapy. *Cancer Res*. 1993;53:2597-2602.
45. Koreth J, Matsuoka K, Kim HT, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med*. 2011;365:2055-2066.

46. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011;364:2119-2127.
47. Eder M, Geissler G, Ganser A. IL-3 in the clinic. *Stem Cells*. 1997;15:327-333.
48. Lindemann A, Mertelsmann R. Interleukin-3 and its receptor. *Cancer Treat Res*. 1995;80:107-142.
49. Talpaz M, Patterson M, Kurzrock R. Sequential administration of IL-3 and Gm-Csf in Bone-Marrow failure patients - a phase-I study. *Blood*. 1994;84:A28.
50. Kurzrock R, Talpaz M, Estrov Z, et al. Phase I study of recombinant human interleukin-3 in patients with bone marrow failure. *J Clin Oncol*. 1991;9:1241-1250.
51. Wu HH, Talpaz M, Champlin RE, et al. Sequential interleukin 3 and granulocyte-macrophage-colony stimulating factor therapy in patients with bone marrow failure with long-term follow-up of responses. *Cancer*. 2003;98:2410-2419.
52. McKenzie AN, Culpepper JA, de Waal Malefyt R, et al. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci U S A*. 1993;90:3735-3739.
53. Wetzler M, Kurzrock R, Estrov Z, et al. Suppressed formation of bone marrow adherent layers derived from acute myeloid leukemia patients after in vitro exposure to interleukin-4. *Leuk Res*. 1997;21:519-527.
54. Whitehead RP, Unger JM, Goodwin JW, et al. Phase II trial of recombinant human interleukin-4 in patients with disseminated malignant melanoma: a Southwest Oncology Group study. *J Immunother*. 1998;21:440-446.
55. Karlen S, De Boer ML, Lipscombe RJ, et al. Biological and molecular characteristics of interleukin-5 and its receptor. *Int Rev Immunol*. 1998;16:227-247.
56. Kurzrock R, Redman J, Cabanillas F, et al. Serum interleukin 6 levels are elevated in lymphoma patients and correlate with survival in advanced Hodgkin's disease and with B symptoms. *Cancer Res*. 1993;53:2118-2122.
57. Seymour JF, Talpaz M, Cabanillas F, et al. Serum interleukin-6 levels correlate with prognosis in diffuse large-cell lymphoma. *J Clin Oncol*. 1995;13:575-582.
58. Seymour JF, Talpaz M, Hagemeister FB, et al. Clinical correlates of elevated serum levels of interleukin 6 in patients with untreated Hodgkin's disease. *Am J Med*. 1997;102:21-28.
59. Hsu SM, Waldron JA, Xie SS, et al. Expression of interleukin-6 in Castleman's disease. *Hum Pathol*. 1993;24:833-839.
60. Dorff TB, Goldman B, Pinski JK, et al. Clinical and correlative results of SWOG S0354: a phase II trial of CNTO328 (siltuximab), a monoclonal antibody against interleukin-6, in chemotherapy-pretreated patients with castration-resistant prostate cancer. *Clin Cancer Res*. 2010;16:3028-3034.
61. Namen AE, Lupton S, Hjerrild K, et al. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature*. 1988;333:571-573.
62. Goodwin RG, Friend D, Ziegler SF, et al. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell*. 1990;60:941-951.
63. Akashi K, Kondo M, Weissman IL. Role of interleukin-7 in T-cell development from hematopoietic stem cells. *Immunol Rev*. 1998;165:13-28.
64. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. *Blood*. 2002;99:3892-3904.
65. Sportes C, Babb RR, Krumlauf MC, et al. Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. *Clin Cancer Res*. 2010;16:727-735.
66. Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev*. 2001;12:375-391.
67. Bar-Eli M. Role of interleukin-8 in tumor growth and metastasis of human melanoma. *Pathobiology*. 1999;67:12-18.
68. Renauld JC. Interleukin-9: structural characteristics and biologic properties. In: Kurzrock R, Talpaz M, eds. *Cytokines: Interleukins and Their Receptors*. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1995.
69. Demoulin JB, Renauld JC. Interleukin 9 and its receptor: an overview of structure and function. *Int Rev Immunol*. 1998;16:345-364.
70. Cortes J, Kurzrock R. Interleukin-10 in non-Hodgkin's lymphoma. *Leuk Lymphoma*. 1997;26:251-259.
71. Akdis CA, Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology*. 2001;103:131-136.
72. Dumoutier L, Renauld JC. Viral and cellular interleukin-10 (IL-10)-related cytokines: from structures to functions. *Eur Cytokine Netw*. 2002;13:5-15.
73. Stasi R, Zinzani L, Galieni P, et al. Detection of soluble interleukin-2 receptor and interleukin-10 in the serum of patients with aggressive non-Hodgkin's lymphoma. Identification of a subset at high risk of treatment failure. *Cancer*. 1994;74:1792-1800.
74. Cortes JE, Talpaz M, Cabanillas F, et al. Serum levels of interleukin-10 in patients with diffuse large cell lymphoma: lack of correlation with prognosis. *Blood*. 1995;85:2516-2520.
75. Du X, Williams DA. Interleukin-11: review of molecular, cell biology, and clinical use. *Blood*. 1997;89:3897-3908.
76. Kurzrock R, Cortes J, Thomas DA, et al. Pilot study of low-dose interleukin-11 in patients with bone marrow failure. *J Clin Oncol*. 2001;19:4165-4172.
77. Lewis VO, Ozawa MG, Deavers MT, et al. The interleukin-11 receptor alpha as a candidate ligand-directed target in osteosarcoma: consistent data from cell lines, orthotopic models, and human tumor samples. *Cancer Res*. 2009;69:1995-1999.
78. Huang G, Yu L, Cooper LJ, et al. Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases. *Cancer Res*. 2012;72:271-281.
79. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med*. 1989;170:827-845.
80. Trinchieri G, Scott P. Interleukin-12: basic principles and clinical applications. *Curr Top Microbiol Immunol*. 1999;238:57-78.
81. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol*. 1998;70:83-243.
82. Ansell SM, Geyer SM, Maurer MJ, et al. Randomized phase II study of interleukin-12 in combination with rituximab in previously treated non-Hodgkin's lymphoma patients. *Clin Cancer Res*. 2006;12(20 Pt 1):6056-6063.
83. Yang ZZ, Grote DM, Ziesmer SC, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Invest*. 2012;122:1271-1282.
84. Trentin L, Zambello R, Facco M, et al. Interleukin-15: a novel cytokine with regulatory properties on normal and neoplastic B lymphocytes. *Leuk Lymphoma*. 1997;27:35-42.
85. Waldmann T, Tagaya Y, Bamford R. Interleukin-2, interleukin-15, and their receptors. *Int Rev Immunol*. 1998;16:205-226.
86. Cruikshank WW, Kornfeld H, Center DM. Signaling and functional properties of interleukin-16. *Int Rev Immunol*. 1998;16:523-540.
87. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med*. 1996;183:2593-2603.
88. Yao Z, Painter SL, Fanslow WC, et al. Human IL-17: a novel cytokine derived from T cells. *J Immunol*. 1995;155:5483-5486.
89. Bazan JF, Timans JC, Kastelein RA. A newly defined interleukin-1? *Nature*. 1996;379:591.
90. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. *Ann N Y Acad Sci*. 1998;856:1-11.
91. Terme M, Ullrich E, Aymeric L, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res*. 2011;71:5393-5399.
92. Gallagher G, Dickensheets H, Eskdale J, et al. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). *Genes Immun*. 2000;1:442-450.
93. Blumberg H, Conklin D, Xu WF, et al. Interleukin 20: discovery, receptor identification, and role in epidermal function. *Cell*. 2001;104:9-19.
94. Pestka S, Krause CD, Sarkar D, et al. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol*. 2004;22:929-979.
95. Dumoutier L, Leemans C, Lejeune D, et al. Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. *J Immunol*. 2001;167:3545-3549.
96. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature*. 2000;408:57-63.
97. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. *Science*. 2002;298:1630-1634.
98. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol*. 2008;26:57-79.

99. Andorsky DJ, Timmerman JM. Interleukin-21: biology and application to cancer therapy. *Expert Opin Biol Ther*. 2008;8:1295-1307.
100. Sarra M, Franze E, Pallone F, et al. Targeting interleukin-21 in inflammatory diseases. *Expert Opin Ther Targets*. 2011;15:695-702.
101. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One*. 2012;7:e30264.
102. Watanabe M, Kono K, Kawaguchi Y, et al. Interleukin-21 can efficiently restore impaired antibody-dependent cell-mediated cytotoxicity in patients with oesophageal squamous cell carcinoma. *Br J Cancer*. 2010;102:520-529.
103. Smyth MJ, Teng MW, Sharkey J, et al. Interleukin 21 enhances antibody-mediated tumor rejection. *Cancer Res*. 2008;68:3019-3025.
104. Li Y, Bleakley M, Yee C. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. *J Immunol*. 2005;175:2261-2269.
105. Li Y, Yee C. IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific $CD8^+$ cytotoxic T lymphocytes. *Blood*. 2008;111:229-235.
106. Li Y, Liu S, Hernandez J, et al. MART-1-specific melanoma tumor-infiltrating lymphocytes maintaining CD28 expression have improved survival and expansion capability following antigenic restimulation in vitro. *J Immunol*. 2010;184:452-465.
107. Parmigiani A, Pallin MF, Schmidtmayerova H, et al. Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells. *Hum Immunol*. 2011;72:115-123.
108. Strbo N, de Armas L, Liu H, et al. IL-21 augments natural killer effector functions in chronically HIV-infected individuals. *AIDS*. 2008;22:1551-1560.
109. Hashmi MH, Van Veldhuizen PJ. Interleukin-21: updated review of Phase I and II clinical trials in metastatic renal cell carcinoma, metastatic melanoma and relapsed/refractory indolent non-Hodgkin's lymphoma. *Expert Opin Biol Ther*. 2010;10:807-817.
110. Schmidt H, Brown J, Mouritzen U, et al. Safety and clinical effect of subcutaneous human interleukin-21 in patients with metastatic melanoma or renal cell carcinoma: a phase I trial. *Clin Cancer Res*. 2010;16:5312-5319.
111. Davis ID, Skrumsager BK, Cebon J, et al. An open-label, two-arm, phase I trial of recombinant human interleukin-21 in patients with metastatic melanoma. *Clin Cancer Res*. 2007;13:3630-3636.
112. Thompson JA, Curti BD, Redman BG, et al. Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. *J Clin Oncol*. 2008;26:2034-2039.
113. Steele N, Anthony A, Saunders M, et al. A phase 1 trial of recombinant human IL-21 in combination with cetuximab in patients with metastatic colorectal cancer. *Br J Cancer*. 2012;106:793-798.
114. Grunwald V, Desar IM, Haanen J, et al. A phase I study of recombinant human interleukin-21 (rIL-21) in combination with sunitinib in patients with metastatic renal cell carcinoma (RCC). *Acta Oncol*. 2011;50:121-126.
115. Dumoutier L, Louahed J, Renauld JC. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J Immunol*. 2000;164:1814-1819.
116. Kotenko SV, Izotova LS, Mirochnitchenko OV, et al. Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J Biol Chem*. 2001;276:2725-2732.
117. Sabat R, Wolk K. Research in practice: IL-22 and IL-20: significance for epithelial homeostasis and psoriasis pathogenesis. *J Dtsch Dermatol Ges*. 2011;9:518-523.
118. Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol*. 2002;168:5699-5708.
119. Jiang H, Lin JJ, Su ZZ, et al. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene*. 1995;11:2477-2486.
120. Caudell EG, Mumm JB, Poindexter N, et al. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. *J Immunol*. 2002;168:6041-6046.
121. Wang M, Tan Z, Zhang R, et al. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J Biol Chem*. 2002;277:7341-7347.
122. Ekmekcioglu S, Ellerhorst J, Mhashilkar AM, et al. Down-regulated melanoma differentiation associated gene (mda-7) expression in human melanomas. *Int J Cancer*. 2001;94:54-59.
123. Ellerhorst JA, Prieto VG, Ekmekcioglu S, et al. Loss of MDA-7 expression with progression of melanoma. *J Clin Oncol*. 2002;20:1069-1074.
124. Chada S, Mhashilkar AM, Ramesh R, et al. Bystander activity of Ad-mda7: human MDA-7 protein kills melanoma cells via an IL-20 receptor-dependent but STAT3-independent mechanism. *Mol Ther*. 2004;10:1085-1095.
125. Cunningham CC, Chada S, Merritt JA, et al. Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol Ther*. 2005;11:149-159.
126. Tong AW, Nemunaitis J, Su D, et al. Intratumoral injection of INGN 241, a nonreplicating adenovector expressing the melanoma-differentiation associated gene-7 (mda-7/IL24): biologic outcome in advanced cancer patients. *Mol Ther*. 2005;11:160-172.
127. Fort MM, Cheung J, Yen D, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*. 2001;15:985-995.
128. Sheikh F, Baurin VV, Lewis-Antes A, et al. Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. *J Immunol*. 2004;172:2006-2010.
129. Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive $CD4^+$ T cells. *Immunity*. 2002;16:779-790.
130. Hisada M, Kamiya S, Fujita K, et al. Potent antitumor activity of interleukin-27. *Cancer Res*. 2004;64:1152-1156.
131. Shimizu M, Shimamura M, Owaki T, et al. Antiangiogenic and antitumor activities of IL-27. *J Immunol*. 2006;176:7317-7324.
132. Airoldi I, Cocco C, Giuliani N, et al. Constitutive expression of IL-12R beta 2 on human multiple myeloma cells delineates a novel therapeutic target. *Blood*. 2008;112:750-759.
133. Yoshimoto T, Morishima N, Mizoguchi I, et al. Antiproliferative activity of IL-27 on melanoma. *J Immunol*. 2008;180:6527-6535.
134. Cocco C, Giuliani N, Di Carlo E, et al. Interleukin-27 acts as multifunctional antitumor agent in multiple myeloma. *Clin Cancer Res*. 2010;16:4188-4197.
135. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol*. 2003;4:63-68.
136. Diegelmann J, Beigel F, Zitzmann K, et al. Comparative analysis of the lambda-interferons IL-28A and IL-29 regarding their transcriptome and their antiviral properties against hepatitis C virus. *PLoS One*. 2010;5:e15200.
137. Dillon SR, Sprecher C, Hammond A, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol*. 2004;5:752-760.
138. Dreuw A, Radtke S, Pflanz S, et al. Characterization of the signaling capacities of the novel gp130-like cytokine receptor. *J Biol Chem*. 2004;279:36112-36120.
139. Kim SH, Han SY, Azam T, et al. Interleukin-32: a cytokine and inducer of TNFalpha. *Immunity*. 2005;22:131-142.
140. Novick D, Rubinstein M, Azam T, et al. Proteinase 3 is an IL-32 binding protein. *Proc Natl Acad Sci U S A*. 2006;103:3316-3321.
141. Nold-Petry CA, Nold MF, Zepp JA, et al. IL-32-dependent effects of IL-1beta on endothelial cell functions. *Proc Natl Acad Sci U S A*. 2009;106:3883-3888.
142. Oh JH, Cho MC, Kim JH, et al. IL-32gamma inhibits cancer cell growth through inactivation of NF-kappaB and STAT3 signals. *Oncogene*. 2011;30:3345-3359.
143. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23:479-490.
144. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*. 2010;10:103-110.
145. Carriere V, Roussel L, Ortega N, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A*. 2007;104:282-287.
146. Baekkevold ES, Roussigne M, Yamanaka T, et al. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol*. 2003;163:69-79.
147. Niedbala W, Wei XQ, Cai B, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*. 2007;37:3021-3029.

148. Towne JE, Renshaw BR, Douangpanya J, et al. Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity. *J Biol Chem*. 2011;286:42594-42602.
149. Kumar S, McDonnell PC, Lehr R, et al. Identification and initial characterization of four novel members of the interleukin-1 family. *J Biol Chem*. 2000;275:10308-10314.
150. Dinarello C, Arend W, Sims J, et al. IL-1 family nomenclature. *Nat Immunol*. 2010;11:973.
151. Nold MF, Nold-Petry CA, Zepp JA, et al. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol*. 2010;11:1014-1022.
152. Rahman P, Sun S, Peddle L, et al. Association between the interleukin-1 family gene cluster and psoriatic arthritis. *Arthritis Rheum*. 2006;54:2321-2325.
153. Chou CT, Timms AE, Wei JC, et al. Replication of association of IL1 gene complex members with ankylosing spondylitis in Taiwanese Chinese. *Ann Rheum Dis*. 2006;65:1106-1109.
154. van de Veerdonk FL, Stoeckman AK, Wu G, et al. IL-38 binds to the IL-36 receptor and has biological effects on immune cells similar to IL-36 receptor antagonist. *Proc Natl Acad Sci U S A*. 2012;109:3001-3005.

Scott M. Lippman and J. Jack Lee

58 Cancer Chemoprevention

Chemoprevention is the use of pharmacologic interventions to reduce the risk of cancer or to treat or reduce the risk of intraepithelial neoplasia (IEN[1]) developing into cancer. As a noninvasive lesion representing an often pathologically discernable intermediate state between normal and malignant tissue, IEN has a substantial cancer risk.[2] The molecular biology of preinvasive carcinogenesis and drug interventions was substantially advanced by translational research of a few pioneering groups, including studies by Hong and colleagues in head and neck carcinogenesis and its response to retinoids.[1,3-7] These early studies presaged the emergence of molecular-targeted approaches, which have not only become a mainstay in therapeutic drug development but are also the major focus of chemopreventive drug development today.[8]

Molecular-Targeted Prevention

Molecular-targeted drug development is based on the concept that neoplasia is a multistep process, which involves accumulating genetic and epigenetic alterations driven by genomic instability, and a multifocal process, which involves field carcinogenesis and clonal spread.[1,9] Hallmarks of these processes include evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, strong replicative potential, and sustained angiogenesis. These molecular alterations and hallmarks can develop in IEN. Major advances in molecular-targeted chemoprevention include the drugs tamoxifen and raloxifene (targeting the estrogen receptor [ER]), finasteride (targeting 5α-reductase), and celecoxib (targeting cyclooxygenase-2 [COX-2][10-16]).

Biomarkers play a major role in all aspects of molecular-targeted chemoprevention, including as (1) molecular targets for identifying new agents; (2) targets to help determine the biologically active doses delivered to tissue; (3) cancer risk, prognosis, or predictive markers for selecting (or stratifying) study patients; (4) endpoints of Phase Ib and Phase II drug activity trials; and (5) surrogate efficacy and toxicity endpoints in Phase III cancer prevention trials. Although receiving keen interest, surrogate biomarkers are extremely complicated and may yet be a long way from validation as primary endpoints of Phase III trials.[17,18] Encouraging results on potential new surrogate endpoint biomarkers are emerging from work on proteomic and genomic profiling in trials of the selective COX-2 inhibitor celecoxib,[19,20] highlighting the convergence of molecular markers in chemoprevention with those of early-detection research. Spira and colleagues[21] were the first to define the reversible and irreversible genetic effects of cigarette smoke using gene expression profiling on human airway epithelial cells. Given that cigarette smoke creates a field of injury throughout the airway, they identified an 80-gene biomarker that distinguishes smokers with and without lung cancer. This biomarker panel had approximately 90% sensitivity for stage I cancer across all subjects and 95% sensitivity when combined with lower airway cell cytopathology, demonstrating a potential cancer-specific airway-wide response to cigarette smoke.[22] Also exciting is the discovery that the miRNA profile derived from serum and plasma of various tissues/organs shows immense promise as a novel noninvasive biomarker for the diagnosis of cancer and other diseases.[23] Because surgical pathology specimens from lung biopsies are stored as formalin-fixed, paraffin-embedded blocks and are widely available, miRNA expression and hypermethylation of genes can be successfully extracted as candidate biomarkers for the early detection of lung cancer.[24] Molecular biomarkers also can be used to confirm IEN response, the importance of which was suggested by genetic abnormalities that persisted at the site of head and neck IEN that had responded completely (clinically and histologically) in a chemoprevention trial.[25,26]

Molecular biomarkers also are used in the emerging field of preventive pharmacogenomics. Germ-line *BRCA2* mutations in people receiving tamoxifen, *SRD5A2* polymorphisms in people receiving finasteride, cyclin D1 polymorphisms in people receiving retinoids, *CYP2C9* genotypes in people receiving nonsteroidal anti-inflammatory drugs

(NSAIDs), and epidermal growth factor receptor (*EGFR*) tyrosine kinase (TK) domain mutations in patients receiving EGFR TK inhibitors (TKIs) are important examples of pharmacogenomic biomarkers.[27-31]

Prevention-Therapy Convergence

Molecular-targeting research is blurring the distinction between malignancy and premalignancy and between cancer therapy and prevention. A new generation of targeted drugs with acceptable therapeutic indices for prevention and therapy is emerging from the molecular study of neoplasia (IEN and cancer), drug effects on relevant pathways, and cancer risk/prognosis.[3] Targeted drugs can move from therapy to prevention (exemplified by tamoxifen, aromatase inhibitors, and EGFR inhibitors) or vice versa (celecoxib). It is likely that tamoxifen both prevented and treated subclinical cancer in the Breast Cancer Prevention Trial (BCPT) and adjuvant breast cancer trials. Always problematical, the distinction in cancer survivors between a second primary tumor (SPT), which is a prevention endpoint, and recurrence, which is a therapy endpoint, has been blurred further by molecular studies in breast and head and neck neoplasia. The distinction between cancer and IEN is blurred in definitively treated oral cancer patients who develop IEN at a very high risk of a new cancer because of genomic instability and loss of heterozygosity (LOH[32,33]). Furthermore, it is very difficult to determine if the new cancer developing in these patients is an SPT or a recurrence. Rigorous clinical determinations of SPT or recurrence following curative treatment of head and neck cancer have been questioned by genetic profiling that revealed substantial molecular ambiguity regarding the origins of the subsequent cancers. For example, more than 50% of the clinically defined SPTs were molecularly determined to be recurrences (i.e., to have genetic profiles consistent with clonal spread of the original tumor[34,35]).

In recent years, the paradigm in which "at-risk" tissue is visualized by autofluorescence has evolved and progressed from the lungs into the oral cavity as well as other major organ sites. New developments coupling autofluorescence with digital imaging/processing have the potential to become an important diagnostic adjuvant and make a significant impact on detection and evaluation of tissue alterations associated with neoplastic development. An exciting new imaging modality recently provided proof-of-principle in the ability of noninvasive optical imaging to accurately identify neoplastic tissue and premalignant lesions.[36-39] Using novel biomedical optics technology, microvascular blood content in the proximity of a neoplasia can be measured and evaluated for field effects and can play a potential role in distinguishing various stages of neoplasia.[40]

Convergent Trial Designs

Two convergent trial designs involving molecular-targeted agents are (1) a Phase I design in which toxicity and pharmacodynamic effects (e.g., optimal biologic doses) are assessed to determine the dose of an agent for subsequent Phase II testing in either prevention or therapy and (2) a therapy design with embedded prevention endpoints (e.g., IEN) for agents with preventive potential based on mechanistic and safety characteristics.[3] The Phase I design can include assessments of pharmacodynamic effects on tumor and surrounding or surrogate tissue. The embedding design can include Phase II or III trials in cancer settings with a prevalent IEN, for example, rectal aberrant crypt foci (ACFs) in single-agent colon cancer trials. ACFs can be identified by magnifying endoscopy (e.g., flexible sigmoidoscopy in the rectum) and are thought to be precursors of adenomas. ACFs often show Apc loss, K-ras mutations, and EGFR and erbB2 upregulation, and the number, size, and dysplastic features of ACFs correlate with the number of adenomas. ACFs appeared to be suppressed by NSAIDs in observational studies, by EGFR TKIs in preclinical studies, and by metformin in an early clinical trial.[41,42] Another embedded convergence approach is to assess at-risk tissue in adjuvant trials—for example, bronchoscopic studies in adjuvant lung cancer trials. A recent study detected *EGFR* TK domain mutations and increased estrogen receptor expression in histologically normal lung tissue surrounding a primary lung adenocarcinoma with *EGFR* mutations.[43,44] This apparent field effect raises important biologic issues and may help identify patients more likely to benefit from adjuvant therapy with EGFR TKIs. Drug activity in high-risk IEN is relevant to the therapy setting; prevention trials in high-risk settings and therapy trials have similar sizes, durations, costs, and ethical considerations (high cancer risk justifies potential adverse drug effects, as does cancer prognosis[45]). The highest risk IENs, such as familial adenomatous polyposis and oral IEN with LOH, are promising settings for convergent drug development.

Short-term trials in patients before a scheduled surgery also can be used for early-phase convergent drug development, as illustrated by recent studies of EGFR TKIs in breast neoplasia. The EGFR TKIs gefitinib and erlotinib reduced cell proliferation in randomized presurgical trials in women with ductal carcinoma in situ or early-stage breast cancer.[46,47] Although not involving therapy, a novel convergent approach is to embed prevention endpoints in a screening study. A recent randomized trial of inhaled budesonide was embedded within a spiral computed tomography (CT) screening study involving high-risk people with peripheral lung nodules (presumed precursors of adenocarcinoma). Although this study yielded negative results, the treatment was well tolerated. This novel trial design was the first formal

clinical assessment of preventive effects on adenocarcinoma precursors in the peripheral airway.[48]

Promising Convergent Targets and Drugs

Many promising targets for cancer prevention and therapy are in preclinical studies related to drugs currently in clinical testing (Table 58-1). Some of the major signaling pathways with promising molecular targets are discussed in the following sections.

EGFR Signaling

EGFR is upstream of several major targets/pathways, including COX-2, PI3K, and vascular endothelial growth factor (VEGF), and has complex interactions with retinoic acid signaling and the IGF axis (discussed later). (EGFR also is upstream of cyclin D1, signal transducer and activator of transcription-3 [STAT3], and Src.) The importance of EGFR as a prevention-therapy target is illustrated in lung carcinogenesis. High *EGFR* (*ErbB1*) gene copy number and protein expression occur in lung IEN and have been associated with a poor prognosis in resected non–small-cell lung cancer. EGFR inhibitors have activity in a mouse lung cancer prevention model and in non–small-cell lung cancer therapy (in association with high EGFR). *EGFR* TK domain mutations (which are associated with EGFR TKI response) have been detected in high-risk nonmalignant lung tissue.[43] EGFR is a potential target for convergent drug development in several sites and, as discussed in the following paragraphs, EGFR signaling has complex pathway interactions and feedback loops that make it very promising for use in combination targeting approaches.

Polyunsaturated Fatty-Acid Metabolic Signaling

Membrane phospholipids including arachidonic acid (AA) and linoleic acid (LA) are converted by a series of enzymes to a variety of eicosanoids, which differ markedly in their biological activities. The cyclooxygenase (COX) pathway leads mostly to the generation of prostaglandins (PGs), prostacyclins, and thromboxanes (TXs), whereas the lipoxygenase (LOX) pathway leads to the formation of leukotrienes, hydroxyeicosatetraenoic acids (HETEs), lipoxins, and hydroxyoctadecadienoic acid (HODEs). These biologically active lipids orchestrate the complex interactions between transformed epithelial cells and the surrounding stromal cells and play crucial roles in chronic inflammation and cancer (as reviewed in Reference 49). The enzymes involved in AA metabolism (COX1/2, 5-LOX, and 12-LOX) have been among the most extensively studied targets for anticancer therapy and prevention. In addition to its other effects, aspirin (a nonselective NSAID) has been shown to be an especially effective chemopreventive agent for colorectal neoplasia, through its actions as an inhibitor of the COX-2 pathway, which is overexpressed in 80% to 85% of colorectal cancers.[50-52] The LOX pathway enzymes (5-LOX, 12-LOX, and 15-LOX-1/2) also have an important role in tumor progression and survival. The 15-LOX-1 enzyme and its products (15-S-HETE and 13-S-HODE) induce apoptosis, and losses of 15-LOX-1 expression and enzymatic activity were the only significant changes in LOX metabolism that related to the loss of cell differentiation and apoptosis in colon cancer cells in vitro and in polyps of familial adenomatous polyposis patients.[53,54] Pharmacologic or genetic restoration of 15-LOX-1 induces apoptosis and suppresses tumorigenesis in vivo. 15-LOX-1 interacts with GATA-6, protein kinase G, histone deacetylase (HDAC), methyltransferase (upstream 15-LOX-1 regulators), and PPAR-δ and -γ (downstream 15-LOX-1 mediators) to induce apoptosis and suppress carcinogenesis.[53-56] 13-S-HODE downregulates PPAR-δ to activate PPAR-γ and induce apoptosis, indicating that polyunsaturated fatty acid oxidative metabolism can influence the balance between PPAR-δ and PPAR-γ. Understanding the roles of prostaglandins and leukotrienes in epithelial-derived tumors and their microenvironment may help to develop cancer biomarkers and chemopreventive and/or therapeutic agents with minimal side effects compared to NSAIDs.

Nuclear Receptor Signaling

Members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors are implicated in a broad spectrum of physiologic and pathophysiologic processes. As well, NRs have widespread anti-inflammatory roles in the cells of the immune system that contribute to the tumor microenvironment. Evidence exists for an increased risk for cancer development among patients with chronic inflammatory diseases such as diabetes or ulcerative colitis. That NRs and their ligands play a prominent role in modulating the microenvironment and inhibiting tumor-promoting inflammation makes them promising therapeutic targets for high-risk populations when used in combinatorial or chemopreventive strategies.[57]

Promising convergent targets also are emerging from studies of retinoid signaling through retinoic acid receptor (RAR) and retinoid X receptor (RXR) types, subtypes, and isoforms.[1,4] Retinoids modulate cell growth and gene expression by activating nuclear RARs and RXRs, each of which

Table 58-1 Molecular Targets and Their Agents in Development for, or Relevant to, Cancer Prevention and Therapy

Molecular Targets	Agents
Prevention and Therapy	
ER-α*	Tamoxifen,* raloxifene,* arzoxifene
5α-reductase**	Finasteride,** dutasteride**
COX-2*	Celecoxib,* rofecoxib
Ornithine decarboxylase	DFMO
p53	INGN201, ONYX-015
5-LOX	Zileuton
Prostacyclin	Iloprost
Aromatase	Exemestane, letrozole, anastrozole
Androgen receptor	Flutamide
PPAR-γ	Rosiglitazone
Retinoic acid receptor/ retinoid X receptor	9-*cis*-Retinoic acid
Retinoid X receptor	Bexarotene
EGFR	Gefitinib, erlotinib, cetuximab
Therapy†	
Farnesyl transferase	Tipifarnib, lonafarnib
mTOR	RAD-001, CCI-779, metformin
DNA methyltransferase	Azacytidine
Histone deacetylase	SAHA
PI3K/Akt	Deguelin, myo-inositol
MMP	Marimastat (broad), matlystatin B (MMP-1), metastat (MMP-2/9)
TRAIL	Apo2L/TRAIL
CDK	Flavopiridol (cdks 4/6,2,1); BMS 387032, seliciclib (cdks 2,1)
HER-2	Trastuzumab
VEGF	Bevacizumab, VEGF trap
VEGFR	Sorafenib, sunitinib, AZD2171, ZD6474, AMG 706, PTK 787
PDGFR	Imatinib, sunitinib, AZD2171, PTK 787
c-KIT	Imatinib, sunitinib, AZD2171, PTK 787
RET	ZD6474, sunitinib, sorafenib, AMG 706
IGF-1R	CP751871, 12, IGFBP3, metformin
FGFR	BIBF1120, BMS 582664
MEK	AZD6244, CI-1040
B-Raf	Sorafenib
Src	Dasatinib, AZD0530
HIF-1α	17-AAG
Proteosome	Bortezomib

*Target and agent involved in U.S. Food and Drug Administration–approved cancer risk reduction or IEN treatment.
**Target and agent involved in established cancer-risk reduction/chemoprevention.
†Therapy targets and agents with potential for chemoprevention.
5-LOX, 5-Lipoxygenase; *CDK,* cyclin-dependent kinase; *DFMO,* difluoromethylornithine; *EGFR,* epidermal growth factor receptor; *ER,* estrogen receptor; *FGFR,* fibroblast growth factor receptor; *HIF-1α,* hypoxia-inducible factor-1 alpha; *IGF-1R,* insulin-like growth factor-1 receptor; *MEK,* mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; *MMP,* matrix metalloproteinases; *mTOR,* mammalian target of rapamycin; *PDGFR,* platelet-derived growth factor receptor; *PPAR-γ,* peroxisome proliferator-activated receptor gamma; *SAHA,* suberoylanilide hydroxamic acid; *VEGF,* vascular endothelial growth factor; *VEGFR,* VEGF receptor.

exists in several isoforms and possesses distinct functions. For example, the RAR-β_2 subtype is a putative tumor suppressor, whereas RAR-β_4 has oncogenic properties. RAR-β_2 suppresses COX-2 expression and frequently is methylated in tobacco-related and other neoplasias. Recent studies have identified a novel RAR-β_2–induced gene, RRIG1, which encodes a cell membrane protein that binds to and inhibits RhoA activity and mediates the effects of RAR-β_2 on cell growth and gene expression.[58,59] These findings highlight molecular pathways involving RAR-β_2, RRIG1, COX-2, and RhoA—all of which are promising convergent targets.

IGF Axis

Targeting the insulin-like growth factor (IGF) axis continues to be an important area of research for both prevention and therapy, as illustrated by recent data in the aerodigestive tract. Elevated levels of IGF-1 and reduced levels of IGF binding protein 3 (IGFBP-3) are associated with increased risk and poor prognosis in lung and other cancers[60]; IGF-1 is a mitogen for a number of neoplastic cells types. The IGF-1 receptor (IGF-1R) is activated during lung carcinogenesis in vitro and in vivo in animals. Targeting IGFR and its downstream pathways (e.g., by the use of IGFBP-3) inhibits survival of premalignant and malignant bronchial epithelial cells and vascular endothelial cells, decreases tumor growth and angiogenesis, and for this reason may be effective for cancer chemoprevention.[61] However, several recent Phase III chemotherapy trials using IGF-1R-targeting antibodies to target late-stage solid tumors have failed to demonstrate signs of clinical efficacy,[62] suggesting that the need to identify potential biomarkers that could help parse out patients who would benefit most is critical. Despite the poor therapeutic outcome with the anti-IGF1R trials, encouraging results from studies targeting the IGF axis in a combinatorial fashion (e.g., STAT3 or IL-6) may be an alternative strategy for chemoprevention.[63]

PI3K/Akt/mTOR Signaling

Targeting the PI3K/Akt/mTOR signaling pathway is another promising approach, especially in the lung. Tobacco carcinogens induce Akt activation and lung carcinogenesis. The Akt pathway is activated in bronchial premalignancy (both proximal airway and alveolar epithelium) in smokers and patients with lung IEN or cancer. Preclinical in vivo studies show that deguelin and myo-inositol have preventive activity in lung tumorigenesis, in part via suppressing the PI3K/Akt pathway, disrupting Hsp90 function, and inhibiting HIF-1α expression.[64-66] The kinase mammalian target of rapamycin (mTOR) is downstream of Akt, and the mTOR inhibitor CCI-779 blocked malignant progression of premalignant lesions with activated mTOR arising in the alveoli of mice that develop lung cancer because of activated K-ras.[67] The mechanism by which CCI-779 inhibited tumorigenesis was unexpected. These lesions were infiltrated with macrophages, shown immunohistochemically to have prominent activation of mTOR signaling. A similar pattern of macrophage infiltration occurred in human alveolar premalignant lesions (atypical alveolar hyperplasia). Treatment with CCI-779 induced apoptosis of macrophages, which coincided with the chemopreventive effect. In vitro, CCI-779 had no effect on LKR-13, a lung adenocarcinoma cell line derived from this mouse, whereas it did induce apoptosis of macrophages, and conditioned media from macrophages directly stimulated the proliferation of LKR-13 cells. In summary, mTOR is activated in lung premalignancy and is required for malignant progression in the lung. This kinase drives tumorigenesis in part through macrophages, a prominent component of the tumor microenvironment, and the antitumor effect of mTOR inhibition required the presence of the tumor microenvironment. These findings have two important implications: mTOR is a potentially important kinase target, and the tumor microenvironment is crucial in malignant progression and a source for novel targets in chemoprevention. An mTOR inhibitor also has reversed Akt-dependent prostatic IEN in transgenic mice.[68]

Metformin belongs to the biguanide class of antidiabetic drugs and activates the LKB1/AMPK axis (mediating glucose and energy homeostasis) and inhibits cancer cell viability through the inhibition of mTOR. Metformin can also downregulate mTOR and subsequent cell growth through AMPK-independent mechanisms[69] (Figure 58-1). A recent study using mouse models of lung cancer to assess the protective effect of metformin suggested two possible mechanisms: decreased levels of circulating insulin and lowered energy stress leading to inhibition of mTOR.[70] Owing to the fact that studies show metformin is associated with a decreased risk of cancer incidence compared with other treatments (such as insulin) among diabetic patients,[71] metformin is rightfully garnering interest for its role in cancer prevention and therapy and supports further testing in the clinical setting.

Looking at new targets of the antineoplastic activities of metformin yields some surprising and unique mechanisms. In paraquat-treated mice, metformin reduced the levels of mitochondrial ROS in an AMPK-independent manner while also reducing DNA double-stranded breaks.[72] A recent compelling study suggests the molecular mechanism by which metformin can elicit its biologic effects in pancreatic cancer stem-like cells (CSCs) is mediated through reexpression of miRNAs and decreased expression of CSC-specific genes.[73] Indeed, these novel mechanisms may help to explain reduced cancer incidence associated with metformin therapy.

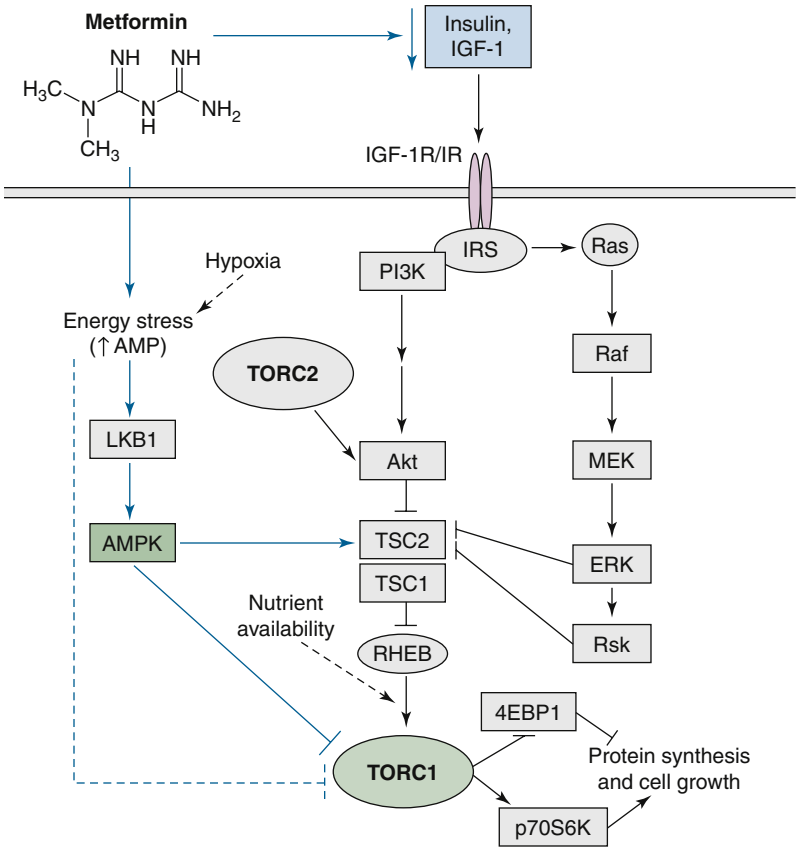


Figure 58-1 mTOR exists in the intracellular complexes TORC1 and TORC2. Growth factor signaling (through PI3K/AKT and ERK/ribosomal S6 kinase [Rsk] signaling) and energy homeostasis (through AMPK) directly lead to TSC2 phosphorylation. In vivo, metformin down-regulates TORC1 via several potential mechanisms including AMPK-dependent and AMPK-independent mechanisms. *IRS,* insulin receptor substrate; *LKB1,* liver kinase B1; *MEK,* mitogen-activated protein kinase. *(Reprinted by permission from the American Association for Cancer Research: Engleman JA, Cantley LC. Chemoprevention meets glucose control.* Cancer Prev Res (Phila). *2010;3:1049-1052.)*

The Angiogenic Switch

The angiogenic switch is a critical regulatory switch (at the level of Ras) within neoplastic cells that targets the endothelium/microenvironment or bone marrow–derived cells recruited to the neoplastic site to reverse endothelial quiescence, thus facilitating pathological angiogenesis. Dysplastic foci and microscopic tumors in various organs can remain undetectable and asymptomatic for years in the absence of inflammation and angiogenesis—both processes key targets for chemoprevention.[74,75] Potential drugs targeting various angiogenic switch regulators such as VEGF receptors (VEGFR including VEGFR1-3), chemokine receptors (CXCR-2 and CXCR-4), miRNAs (e.g., miR-132), and matrix metalloproteinases (e.g., MMP9) can be strategized according to risk to optimize the blockage of vascularization of incipient tumors. Some of these drug targets are under clinical development in therapy as well as for prevention.[76,77]

Combinations and Multiple Targets

Drugs designed to target a single pathway cannot usually combat multigenic diseases such as cancer. Combination drugs that affect multiple targets simultaneously are better at manipulating complex disease systems and are less likely to develop drug resistance. This multidrug treatment modality has become the standard of care in many important therapeutic areas. To that end, cancer chemoprevention using low-dose combinations of different agents instead of a single agent has been suggested to synergistically enhance the preventive effect with less toxicity and fewer side effects (as reviewed in Reference 78). Several promising combinations are listed in Table 58-2, and data supporting a few of these combinations are cited here. Methyltransferase inhibitors plus HDAC inhibitors have been shown to be highly active in vitro and in suppressing lung tumorigenesis in vivo.[79] Many studies support COX-2-inhibitor combinations, and there is extensive preclinical data suggesting that COX-2 inhibition is an attractive target for overcoming resistance to EGFR inhibition. COX-2 inhibitors plus EGFR inhibitors (e.g., in the colon) or aromatase inhibitors (in the breast) involve positive feedback loops. COX-2 inhibitors also block prostaglandin activation of EGFR and induction of aromatase, possibly allowing lower doses and less toxicity of each agent.[80,81] In a recent report, HDAC inhibitors can increase the sensitivity of oral adenomatous squamous carcinoma cells to EGFR inhibitors (possibly via effects on the epithelial-to-mesenchymal transition).[82]

Table 58-2 Promising Molecular-Targeted Combinations for Prevention and Therapy

EGFR inhibitors with:
COX-2 inhibitors
RXR agonists
IGF-1R inhibitors
mTOR inhibitors
VEGF/VEGFR inhibitors
HDAC inhibitors
PPAR-γ agonists
STAT3 inhibitors
COX-2 inhibitors with:
5-LOX inhibitors
12-LOX inhibitors
15-LOX modulators
Aromatase inhibitors
IGFBP-3
IGFBP-3 with:
FTase inhibitors
PI3K/Akt inhibitors
HDAC inhibitors with:
DNMT inhibitors
PPAR-γ agonists
DNMT inhibitors with:
RAR agonists
IGFBP-3

5-LOX, 5-lipoxygenase; *COX-2*, cyclooxygenase-2; *DNMT*, DNA methyltransferase; *EGFR*, epidermal growth factor receptor; *FTase*, farnesyltransferase; *HDAC*, histone deacetylase; *IGF-IR*, insulin-like growth factor-1 receptor; *IGFBP-3*, IGF binding protein-3; *mTOR*, mammalian target of rapamycin; *PI3K*, phosphoinositide 3-kinase; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma; *RAR*, retinoic acid receptor; *RXRs*, retinoid X receptors; *STAT3*, signal transducer and activator of transcription-3; *VEGF*, vascular endothelial growth factor; *VEGFR*, VEGF receptor; *DNMT*, DNA methyltransferase.

Biomarker Cancer Risk Models

The identification of high-risk IEN is a major priority for targeted preventive drug development. This work can include the identification of molecular aberrancies in exfoliated cells and the use of novel imaging technologies. Genetic instability and clonal selection create the risk of cancer development and can be marked by LOH. The 3-year oral cancer risk of oral IEN with LOH at 3p14 and/or 9p21 is 25%.[6,83-88] Genes implicated in this cancer risk include the *FHIT* tumor suppressor gene (found at 3p14) and the p16/p15/p14 tumor suppressor genes (found at 9p21). The cancer risk increases to at least 35% with the addition of LOH at any other site of a known or candidate tumor suppressor gene (e.g., *TRAIL-R1* and *TRAIL-R2* at 8p21 and p53 at 17p13, respectively). The cancer risk associated with LOH in oral IEN has been confirmed by the consistent results of three independent groups. Recently, the oral IEN risk predictor model was further refined with the addition of another two markers (loci on 4q/17p).[85] LOH at 3p and/or 9p in IEN associated with curatively treated oral cancer has a 69% risk of a new oral cancer in 3 years. There are various biomarker-based models for predicting the oral cancer risk of oral IEN that integrates p53, LOH, and chromosomal polysomy,[89] or combines LOH and nuclear phenotypic score (as measured by the Quantitative Tissue Phenotype Imaging System),[90] or uses gene expression profiling.[91] Recent reports outline similar biomarker panels for the cancer risk of esophageal IEN (Barrett's esophagus[92-95]).

Novel risk assessment models are emerging from the joint efforts of neoplasia biology (e.g., to identify somatic genetic alterations) and molecular epidemiology (to identify constitutional genetic alterations). This work demonstrates that studies of a single gene or signaling pathway can identify germline polymorphisms for assessing risk and carcinogen susceptibility and can recognize epigenetic or genetic events for early detection and prognosis. These studies can also help in understanding the mechanisms of preventive drug response or resistance. Genes first explored for aberrations in tumors have been explored later for germline aberrations contributing to cancer risk and vice versa, providing new targets for cancer prevention. For example, germline type-II 5AR gene (*SRD5A2*) alterations have been associated with cancer risk, somatic *SRD5A2* mutations have been associated with carcinogenesis, and the SRD5A2 protein is the target of finasteride for preventing prostate cancer.[13]

Breast Cancer Risk Models

Current guidelines for breast cancer risk assessment are predominantly based on a modified Gail model as well as a modified Rosner and Colditz model. In 1989, a landmark study by Gail and colleagues[96] described the first prediction model, which estimated the risk of developing breast cancer (in a given time period) of an American woman at a given age based on six risk factors: age, age at first live birth, age at menarche, history of breast cancer in first-degree relatives, number of previous breast biopsies, and history of atypical hyperplasia. The Gail model was subsequently modified several times to include larger, more diverse cohorts (using U.S. and international registries) and other unconsidered risk factors such as mammographic breast density and hormonal replacement therapy as additional predictive factors. The Rosner and Colditz model[97] developed in 1996 was based on the assumption that relevant breast tissue aging variables played a larger role in risk assessment. However, that model underwent numerous modifications and validations and later included serum estradiol, which improved discriminatory accuracy. These newer models and others offered relatively higher discriminatory accuracy when compared to the original Gail and Rosner-Colditz models. However, a recent systematic review of breast cancer prediction models reports that, despite the substantial efforts invested in developing prediction models, only modest improvements in discriminatory power were realized, because observer bias and variability typically dilute the predictive and discriminative abilities of the model.[98] Nonetheless, the U.S. Preventive Services Task Force recommends breast cancer risk estimation for all

women considering chemoprophylaxis.[99] The Gail model has been shown to reliably predict risk at the population level, but its discriminatory accuracy at the individual level is modest. Thus, future prospective studies incorporating variables that are easily measured and readily available in routine practice will help minimize measurement bias, and using lessons learned from these prior and ongoing efforts will be useful for improving prediction models of breast cancer incidence and the design of successful intervention trials.[100]

Lung Cancer Risk Models

Lung cancer (LC) is the leading cause of cancer-related deaths in the United States. Although tobacco smoking accounts for the majority of LC, approximately 10% to 15% of patients with LC in the United States are lifelong never smokers. The World Health Organization estimates that 25% of LC worldwide occurs in never smokers.[101] Although cigarette smoking continues to impose substantial health and economic burdens on society, the development of reliable risk prediction tools for estimating lung cancer probability by smoking status holds immense public health implications, especially in light of the increased incidence of LC in the never-smoker cohort. However, there are daunting challenges for estimating LC risk, such as determining the risk factors for the 15% of LCs that occur in lifetime never smokers and identifying ever (former and current) smokers who have the highest risk of developing LC. The ability to estimate an individual's absolute risk could be used to guide preventive interventions and to motivate modifications in lifestyle and behavior in the ever-smoker cohort. A few risk-prediction models have been developed in the past decade to estimate an individual's absolute LC risk. The shared risk factors among these models include smoking duration and occupational exposure to asbestos, but differences surface when including family history or lung-related comorbidities.[102] Although the risk models for estimating LC are predominantly smoking-based, Foy and colleagues[103] were able to fit a two-stage clonal expansion (an essential stage in carcinogenesis) model to predict LC risk based on gender and smoking history. Their fitted model (assuming the requirement of two unique mutations) was validated against the control (placebo) arm of the CARET cohort (beta-CArotene and Retinol Efficacy Trial; lung cancer incidence endpoint), where it demonstrated accuracy in predicting the number of LC deaths observed during the trial (after adjusting for healthy volunteers effect). By using data from control subjects in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO), Tammemagi and associates developed and tested predictive models for the general population and a subcohort of ever smokers. These models were externally validated in PLCO intervention arm participants,[104] and although the models demonstrated high predictive accuracy when applied for subsamples of women, men, whites, and nonwhites separately, the models may not be generalizable to other populations because of the average higher socioeconomic status of the PLCO study participants. However, in developing LC risk prediction models with increasing accuracy, these newer models might help reduce LC mortality by identifying current smokers at high risk who might benefit from intensive smoking cessation programs.

Chemoprevention Trials

Nonmolecular-Targeted Trials

Although too toxic for long-term use, high-dose isotretinoin provided the proof-of-principle of chemoprevention in a randomized trial showing that it prevented second primary cancers in patients after definitive treatment of a first head or neck cancer.[105,106] A subsequent trial of low-dose isotretinoin unfortunately did not reduce second primary cancers, recurrences, or mortality in head/neck cancer[107] and lung cancer.[108] These nontargeted trials did help develop many potential targets discussed previously (Promising Convergent Targets and Drugs). Despite consistent epidemiologic data suggesting that β-carotene is associated with a lower risk of lung cancer, β-carotene supplements alone or combined with vitamin A or E increased lung cancer risk in two randomized controlled trials (RCTs) in nearly 50,000 smokers and/or asbestos workers.[109,110] These RCTs suggested that harm correlated with smoking intensity: no evidence from RCTs indicated that β-carotene increased lung cancer risk in nonsmokers, former smokers, or moderate (less than one pack per day) smokers. Another large-scale, nutrient approach RCT (based on prior epidemiologic and laboratory evidence and significant secondary endpoints) testing the whether selenium and vitamin E alone or together decrease prostate cancer (SELECT; Selenium and Vitamin E Cancer Prevention Trial) showed that neither selenium nor vitamin E prevented prostate cancer in a heterogeneous population of healthy men.[111] The longer follow-up of SELECT participants yielded even more dire results: healthy men who took a common dose and formulation of vitamin E alone had a significantly increased risk of prostate cancer.[112] Notwithstanding the generally negative results with vitamin and mineral supplements in developed countries, the combination of β-carotene, vitamin E, and selenium significantly reduced the incidence of gastric cancer and all cancers as well as mortality from cancer in a randomized trial involving 29,581 people in Linxian, China, possibly reflecting the restoration of healthful nutrient levels in an undernourished population.[113,114]

The only proven chemopreventive approach for Barrett's esophagus is photodynamic therapy (PDT). A recent RCT found that PDT doubled the rate of regression of high-grade disease to a lower grade of dysplasia or normal-appearing epithelium (77% vs. 39%) and halved the rate of cancers (13% vs. 28%) at 2 years.[115,116] The PDT group had a rate of adverse events of 94% (vs. 13% in a control group). Complications of PDT in this trial included mild phototoxicity (68%) and significant stricture formation (36%) as well as vomiting, chest pain, constipation, and pyrexia. In mid-2003, the U.S. Food and Drug Administration (FDA) approved and granted orphan drug designation to a photosensitizing porphyrin mixture (Photofrin) in conjunction with PDT for ablation of high-grade disease in patients with Barrett's esophagus who are unable or unwilling to undergo esophagectomy.

Several RCTs of the NSAID aspirin presaged the COX-2–specific targeting trials discussed in Molecular-Targeted Trials. Aspirin (325 mg/d) in 635 colorectal cancer survivors significantly reduced the number of patients with incident adenomas and caused a significant delay in the time to the first adenoma (vs. placebo[117]). An RCT of low-dose (81 mg/d) and high-dose (325 mg/d) aspirin in 1121 patients with prior adenomas showed that only low-dose aspirin reduced the risk of adenomas (especially advanced adenomas[118]). A secondary analysis of the Physicians Health Study (PHS) (involving 22,071 U.S. male physicians) found that aspirin (325 mg every other day) for an average of 5 years did not reduce colorectal cancer risk but slightly (and nonsignificantly) reduced adenomas.[119] The Women's Health Study (WHS) (involving 39,876 U.S. women) found that low-dose aspirin (100 mg every other day) for an average of 10.1 years did not reduce the risks of colorectal cancer or all or other cancers. The International CAPP Consortium recently conducted the largest reported clinical trial in familial adenomatous polyposis (FAP) patients and reported that a daily aspirin (600 mg/d) regimen for more than 1 year resulted in a nonsignificant reduction in polyp load (number and size), lending additional support for an anticancer benefit of aspirin.[120] In addition, a meta-analysis of four RCTs that evaluated aspirin for the secondary prevention of colorectal adenomas indicated that aspirin reduces the risk of recurrence in individuals with a history of these lesions.[121]

Calcium modestly reduced the risk of sporadic adenomas in two randomized trials but did not reduce colorectal cancer in a large-scale trial of calcium plus vitamin D.[122] The incidence of colorectal cancer also was reduced by 37% in the Women's Health Initiative randomized trial of combined estrogen and progestin (vs. placebo) in 16,608 randomized postmenopausal women, but at the expense of an absolute increase in adverse cardiovascular outcomes. In carriers of Lynch syndrome, the most common form of hereditary colorectal cancer, the CAPP2 study (RCT of aspirin chemoprevention with cancer as the primary endpoint) reported substantial protection by aspirin (600 mg/d for a mean of 25 months) against colorectal cancer, making a clear case for the prescription of aspirin to this high-risk group.[123] As well, a pooled analysis of individual patient data from RCTs of daily aspirin versus control for the prevention of vascular events in the United Kingdom bore out aspirin's already known anticancer benefits.[124]

Although, strictly speaking, chemoprevention involves specific compounds (including extracted or synthetic nutrients), foods in the diet also can influence the risk of cancer. Epidemiologic studies of food and diet are conflicting, and randomized trials do not indicate that reduced dietary fat is associated with a reduced risk of breast cancer or that diets high in fruits and vegetables or fiber are independently associated with reduced risk of colorectal neoplasia.[125,126] In a randomized (noncomparative) Phase II trial in high-risk Chinese patients with esophageal dysplastic lesions, lyophilized strawberry powder (60 g/d for 6 months) significantly reduced the histologic grade of patients' precancerous dysplastic lesions as well as protein expression of localized biomarkers, suggesting the protective effects of small fruits and vegetables.[127] Despite disappointing results from recent large-scale randomized prevention trials such as SELECT, results from clinical cancer prevention trials using broccoli sprout–derived beverages for detoxification of airborne pollutants in Qidong, China,[128] and green tea extract to prevent oral cancer in the United States[129] have provided proof-of-principle in a clinical cancer prevention setting and lay the groundwork for future investigations.

Molecular-Targeted Trials

SERMs and Aromatase Inhibitors

The BCPT compared the selective estrogen receptor (ER) modulator (SERM) tamoxifen (20 mg/d) with placebo in preventing breast cancer in 13,388 women at a higher-than-average risk of breast cancer. At a median follow-up of 55 months, tamoxifen reduced the incidence of invasive breast cancer by 49% and noninvasive breast cancer by 50%.[10] These risk reductions were similar for all age and risk groups and were limited to ER-positive tumors. Tamoxifen also reduced the risk of osteoporotic fractures but increased the risk of endometrial cancer, thromboembolism, and cataracts. There was no effect on cardiovascular disease or mental function. Another major tamoxifen prevention trial, the International Breast Cancer Intervention Study (IBIS)-I, found a significant 32% risk reduction with tamoxifen (vs. placebo) in 7410 randomized women.[130] Tamoxifen (20 mg/d for 5 years) received supplemental approval from the FDA for reducing the risk of breast cancer in premenopausal and postmenopausal women with a 5-year predicted risk of at least 1.66%.

The FDA also has approved tamoxifen to reduce the risk of contralateral cancer in breast cancer patients or of invasive breast cancer in women with ductal carcinoma in situ.

Based on suggestive breast-cancer-risk-reduction data from trials in older women to prevent or reduce osteoporosis, the Study of Tamoxifen and Raloxifene (STAR) randomly tested the SERM raloxifene (60 mg/day) against FDA-approved tamoxifen (20 mg/day) for 5 years for reducing the risk of invasive breast cancer and other disease outcomes in 19,747 postmenopausal women (with increased 5-year breast cancer risk). This large, definitive prevention trial showed that raloxifene was equivalent to tamoxifen in effects on invasive breast cancer, had a higher number of noninvasive breast cancers, and had fewer uterine cancers.[11,12] The two drugs were equivalent in effects on other invasive cancer sites, ischemic heart disease events, stroke, and osteoporotic fractures. Raloxifene was associated with fewer cases of pulmonary emboli and deep vein thrombosis and was previously FDA approved and widely used for the prevention and treatment of osteoporosis in postmenopausal women. Raloxifene is now FDA approved based on the results of the STAR trial for reducing the risk of invasive breast cancer.

Tamoxifen and raloxifene illustrate the critical issue of risk-benefit profile for effective, acceptable chemoprevention. Tamoxifen in BCPT reduced invasive breast cancer by 49% (noninvasive by 50%)—however, at the expense of a 2.5-fold increased risk of endometrial cancer and threefold increased risk of pulmonary embolism. Raloxifene in STAR was associated with 38% less endometrial cancer and 36% less pulmonary embolism than was tamoxifen and an equivalent low rate of invasive breast cancer in STAR. Therefore, raloxifene improves the risk-benefit profile in women at risk of invasive breast cancer. A model of these comparative risk-benefit profiles is presented in Figure 58-2. Tamoxifen had

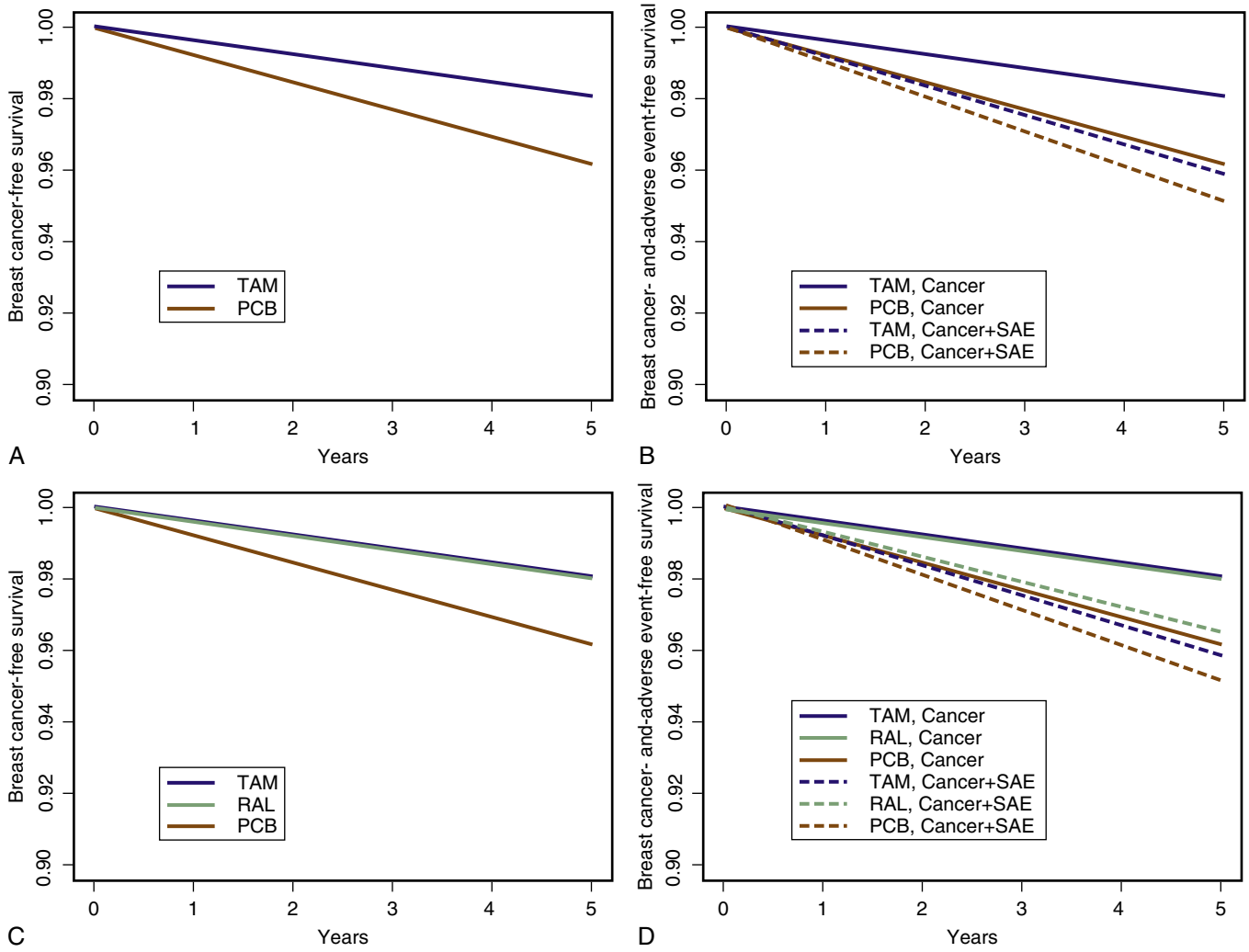


Figure 58-2 Risk-benefit comparisons for tamoxifen (TAM) and raloxifene (RAL) versus placebo (PCB) in breast cancer prevention. (A, B) Comparison of TAM versus PCB without and with the consideration of severe adverse events (SAEs) including the increased risk of endometrial cancer and embolic/vascular events. **(C, D)** Comparison of TAM versus RAL versus PCB without and with the consideration of SAEs. The assumed hazard rates for PCB, TAM, and RAL with the consideration of SAEs are 0.0097, 0.0082, and 0.00687, respectively. These hazard rates are based on data from the primary reports of the Breast Cancer Prevention Trial and Study of Tamoxifen and Raloxifene.

a 14.8% reduced risk overall (including cancer and severe adverse events [SAEs] vs. placebo), and raloxifene had a 29.9% reduced overall risk (including cancer, SAEs, vs. placebo; see Figure 58-2, *D*). SAEs are a major risk of chemoprevention itself, and raloxifene illustrates how to reduce this risk through reducing SAEs. Another way to counter this risk is by identifying target populations with very high cancer risks (discussed earlier in this chapter) who are more SAE tolerant.

Aromatase inhibitors produced greater reductions than tamoxifen in contralateral breast cancer incidence in adjuvant RCTs[131] with fewer endometrial cancer and thromboembolic events and are under active study in prevention. Although less toxic than tamoxifen, these agents have increased risks of osteoporosis and fatal myocardial infarctions, which require further examination in long-term outcome data. A recent double-blinded RCT using exemestane in postmenopausal women at high risk for breast cancer reported a 65% reduction in the diagnosis of invasive breast cancer in women after a median follow-up of 3 years. Exemestane was also associated with no serious toxic effects, supporting its use as an option for risk reduction in postmenopausal women with a high risk of breast cancer.[132]

Large randomized prevention trials show that SERMs can reduce the risk of ER-positive but not ER-negative breast cancer. Extensive data (non-RCT prevention trials) on aromatase inhibitors suggest that these agents will also prevent ER-positive and not ER-negative disease. Three such approaches are RXR agonists, COX-2 inhibitors, and EGFR TKIs, all of which can prevent ER-negative breast cancer in animal models.[133-135] Recent encouraging results from clinical Phase II/III trials demonstrate the utility of anti-EGFR therapies in both adjuvant and neoadjuvant settings.[136-138]

5α-Reductase Inhibitors

The Prostate Cancer Prevention Trial (PCPT) tested finasteride (5 mg/day), which inhibits type II 5α-reductase, the enzyme that converts testosterone to the more potent androgen dihydrotestosterone, for 7 years (vs. placebo) in 18,882 men 55 years of age or older who had a normal digital rectal examination and prostate-specific antigen (PSA) level. Finasteride reduced the 7-year prostate cancer prevalence by 24.8% and reduced high-grade prostatic IEN and benign prostatic hypertrophy (BPH), but it also increased the rates of sexual dysfunction and high-grade prostate cancer.[12] Secondary data from a trial of the dual (type I and II) 5α-reductase inhibitor dutasteride in BPH suggested that this relative of finasteride could also reduce the risk of prostate cancer (both are FDA approved for treating BPH and reducing the risk for urinary retention, but not for the prevention of prostate cancer). These and other data led to a prospective trial of dutasteride to prevent prostate cancer in men with an elevated PSA (between 2.5 and 10 ng/mL) and a negative 6- to 12-core prostate biopsy (REDUCE trial[139]).

In men who are at high risk for prostate cancer, the REDUCE trial demonstrated that men on the dutasteride arm had a 23% overall reduction of being diagnosed with biopsy-detected prostate cancer compared to placebo. This reduction was due to a decreased incidence of lower risk forms of prostate cancer (Gleason score of 6 or lower). However, analogous with the PCPT trial, dutasteride (0.5 mg/d for 4 years) was associated with increased risk of being diagnosed with a more serious form of prostate cancer (high-grade prostate cancer; Gleason score 8 to 10).[140] One interpretation of this result is that although treatment with dutasteride suppressed PSA production, this decrease in PSA levels is the result of shrinkage of benign prostatic tissue and may delay the diagnosis of high-grade prostate cancer until it may be difficult to cure.[141] Despite this increased risk of high-grade prostate cancer, the FDA still states that dutasteride remains safe and effective for their approved indications.

COX-2 and Prostacyclin Inhibitors

A small RCT of celecoxib in FAP patients showed that high-dose celecoxib (400 mg twice daily) for 6 months reduced the number of colorectal adenomas (polyps) compared with placebo (28 vs. 4.5%, $P = .003$[13]). Two secondary endpoints were a reduction in polyp burden and a modest (14%) reduction in difficult-to-resect duodenal polyps. This result led to FDA approval (via accelerated approval) of celecoxib as an adjunct to standard FAP therapy. The primary follow-up study (required by the FDA for drugs approved under the accelerated mechanism) is designed to prevent adenomas in young patients with genotypic familial adenomatous polyposis (Apc mutation carriers) but not yet expressing the phenotype. Three large placebo-controlled RCTs have found that COX-2 inhibitors significantly reduced sporadic adenomas. Rofecoxib (25 mg/day) produced a 25% reduction but also increased serious cardiovascular events, leading the manufacturer of rofecoxib to voluntarily withdraw it from the world market.[142] A large RCT of celecoxib at 200 mg twice daily (b.i.d.) and 400 mg b.i.d. versus placebo found adenoma rates of 61% (placebo), 42% (200 mg b.i.d.), and 37% (400 mg b.i.d.) at 3 years.[15] Another large RCT found that celecoxib at 400 mg/d reduced the 3-year cumulative rate of adenomas by 36% (vs. placebo). Both celecoxib RCTs found greater reductions in advanced than in less advanced adenomas and, unfortunately, increases in celecoxib-associated cardiovascular events.[15,143] On June 8, 2012, approval of the FAP indication for celecoxib was withdrawn by the FDA as requested by the manufacturer (Pfizer).[144] Yet, celecoxib continues to be marketed worldwide while being monitored carefully for cardiovascular safety.

In light of the adverse cardiovascular events, a recent study suggests that patients with a low baseline cardiovascular risk and low serum levels of high-sensitivity C-reactive protein (a circulating inflammatory marker) are at low risk for celecoxib-related SAEs in the treatment of colorectal cancer. These indices may be useful for selecting patients with a favorable risk-benefit profile for celecoxib chemoprevention for personalized therapy.[145]

Approximately 90% of lung cancer cases are attributable to tobacco smoking. Although smoking cessation does not remove the significant risk for lung cancer in former smokers, this risk decreases over time with continued abstinence. Nevertheless, effective chemopreventive agents are still needed. Two recent RCTs show encouraging results in lung cancer chemoprevention in former smokers. Six months of celecoxib regimen (400 mg b.i.d.) in a former-smoker cohort resulted in a decrease in bronchial Ki-67 labeling index (a cellular marker for proliferation).[146] Another RCT using the oral prostacyclin analog iloprost was the first to show statistically significant improvement in bronchial histology (i.e., regression of higher-grade lesions) in the airways of former but not current smokers, demonstrating the strength of the clinical signal provided by this observation and identifying iloprost as a promising chemopreventive agent in preventing lung cancer in former smokers.[147]

Vaccines

Vaccinating children against hepatitis B virus (HBV) has dramatically reduced the incidence and mortality of liver cancer in Taiwan.[148,149] Clinical trials have shown 85% to 95% efficacy in preventing chronic HBV infection, and this response rate can reduce the prevalence of chronic HBV infection to less than 1% in children living in HBV-endemic regions. Properly administered, the effectiveness of the HBV vaccine appears to extend into early adulthood.[150] Estimates from 2002 are that 84% of the world's countries now routinely provide the vaccine.

Infection by human papillomavirus (HPV) includes a spectrum of benign and malignant diseases and is a major worldwide public health concern. Genital HPV is the most common sexually transmitted infection in the United States, and although the majority of infections manifest with no clinical symptoms and are self-limiting, persistent infection with oncogenic types can cause cervical cancer in women. A subset of HPV types (HPV-16 and -18) is the main cause of virtually all cervical cancers as well as other noncervical cancers such as anal, vulvar, and oropharyngeal. The rate of cervical cancer has decreased in the United States because of the widespread use of the Pap smear, yet in certain countries where screening is not routine, the incidence of cervical cancer in women is on the rise.[151] In 2006, the FDA approved the quadrivalent HPV (types 6, 11, 16, and 18) recombinant vaccine (Gardasil) for the vaccination of females 9 to 26 years old for the prevention of cervical cancer, cervical adenocarcinoma in situ, and high-grade cervical, vulvar, and vaginal IEN. The efficacy of the vaccine was studied in four RCTs enrolling 20,541 females (as reviewed in Reference 152). The vaccine was 100% effective for preventing HPV-16– or -18–related cervical, vulvar, and vaginal IEN. This vaccine may reduce the incidence of cervical cancer and the 300,000 deaths it causes worldwide each year, not to mention the other HPV-related diseases such as oropharyngeal cancers.

A distinct form of oropharyngeal squamous cell carcinoma (OPC) is principally caused by HPV and is increasing in incidence among men in the United States. From 1988 to 2004, the population-level incidence of HPV-positive OPC increased by 225% and is expected to exceed the yearly number of cervical cancers by the year 2020. However, vaccine efficacy against oral HPV infections is not known, and therefore vaccination cannot be recommended as a first-line prevention of OPC.[153,154] Interestingly, the incidence of and mortality from this disease are higher in blacks than in whites. HPV-positive OPC patients have significantly better outcomes (vs. HPV-negative) with disease-free survival significantly greater in whites than in blacks. An analysis of overall survival in a retrospective and prospective cohort treated curatively for this disease revealed a racial survival disparity owing to the low prevalence of HPV infection in black OPC patients.[155] However, this outcome disparity data based on race for patients with locally advanced OPC requires confirmation in future analysis.

Vaccines targeting *Helicobacter pylori* are also under development. The public health implications of this work are substantial in view of the fact that *H. pylori* is the major cause of gastric cancer, the fourth most common cancer and second most common cause of cancer death in the world. An estimated 50% of the world's population harbor *H. pylori*, and more than 335,000 deaths resulted from *H. pylori*–caused gastric cancer in 2000.

The burden of *H. pylori*–associated diseases is high in Latin American countries, where resources are low and populations are diverse. In a different approach to address population-based interventions, an RCT of a Latin American population compared the effectiveness of four-drug regimens given concomitantly or sequentially with that of a standard 14-day regimen of triple therapy. In contrast to data from European and some Asian regions where resources are more plentiful and accessible, this study in *H. pylori*–positive patients showed that the probability of *H. pylori* eradication was higher for 14-day standard triple therapy. It was more effective than the 5-day concomitant or 10-day sequential four-drug regimen and shows that eradication of this

infection slows or reverses the progression of premalignant histological lesions.[156] That infections (viral, bacterial, and parasitic) have been recognized as a major cause of cancer worldwide underscores the need for prevention and treatment that can be practically implemented in developing countries where the prevalence of infection-related cancer remains high.[157]

Tumor-antigen–specific vaccines are promising immunopreventive approaches.[158-160] Stimulating an immune response to a specific neoplasia may last a long time and avoid the need for the extended frequent dosing required with cancer treatment or prevention drugs. Animal model studies have stimulated great interest in testing these vaccines for treating or reducing the risk of IEN and thus preventing cancer. Immunodeficient mice develop spontaneous tumors and are more susceptible than are immunocompetent mice to carcinogen-induced tumors, revealing the importance of the host immune response for combating cancer development. Tumor-specific vaccines have demonstrated far greater activity in IEN than cancer in animals, and these vaccines are relatively inactive against advanced cancer clinically. Human host immunosurveillance for IEN is receiving strong support from recent studies in patients showing immune response against the premalignancy monoclonal gammopathy of unknown significance (a clonal expansion of transformed plasma cells that is a precursor of multiple myeloma). These data support the hypothesis that we can boost normal host immune response in IEN with vaccines, and there is intense interest in using vaccines to target IEN. HER2/neu in breast carcinogenesis and MUC1 (e.g., in pancreatic and colorectal IEN) are promising targets for convergent vaccines. Tumor-specific vaccine approaches also may be promising for treating early cancer.[161]

Conclusion

The major direction of chemoprevention is molecular-targeted drug development, which is moving forward with advances in the biology of neoplasia (IEN and cancer), drug effects on relevant targets and pathways, and cancer risk.[162,163] With apparently, in general, lesser toxicity than standard therapy drugs and greater therapeutic activity than many prevention drugs, these agents herald an era of convergent (prevention-therapy) drug development, especially in the area of advanced IEN and early cancer[164,165] (Table 58-3).

Table 58-3 Targeted Agents with Established Cancer Risk-Reducing Effect

Intervention (Year)	Cancer Prevented
Hepatitis B vaccine (1997)	Liver cancer
Tamoxifen (1998) Raloxifene (2007)	Breast cancer Breast cancer
Human papillomavirus vaccine (2006)	Cervical cancer
Finasteride, dutasteride (2004)	Benign prostatic hyperplasia Prostate cancer (Gleason score ≤6)
Celecoxib, rofecoxib (1999)	Precancerous familial adenomatous polyps

Clinical oncology is expanding to the management of cancer risk (tamoxifen, raloxifene, and aromatase inhibitors for breast cancer risk) and IEN (celecoxib for familial adenomatous polyps and tamoxifen for ductal carcinoma in situ). The future also promises to bring a substantial acceleration in the drug development process from discovery to approval, which now takes 10 to 15 years for either a preventive or therapy drug. Merging the silo of new prevention drugs with that of new therapy drugs will potentially cut overall development time in half. The loss of drugs with prevention potential because they fail in therapy testing would cease in the setting of convergent, simultaneous prevention-therapy development. Because many molecular targets or pathways altered in neoplasia also are altered in other aging-related diseases such as atherogenesis, osteoporosis, arthritis, and neurodegenerative diseases, an important new direction of molecular-targeted chemoprevention is the development of agents targeting these shared (as well as distinct) alterations in carcinogenesis and other chronic disease processes.[1,164]

Cancer risk assessment and prevention are without doubt linked by their goal to definitively predict progression to cancer and intervene to reduce risk of cancer incidence and mortality. Extraordinary advances in cancer genomics and epigenomics have the potential to target personalized interventions aimed at the highest risk patients most in need of prevention. Accurate cancer risk biomarker discovery can also preclude unnecessary interventions for low-risk patients, offering them reassurance. These advances are paralleled by an increased understanding of the mechanisms of premalignant conditions and acquired therapeutic resistance that will undoubtedly improve and personalize strategies for cancer management and control—strategies that promise dramatic reductions in cancer mortality.[166]

Key References

8. Abbruzzese JL, Lippman SM. The convergence of cancer prevention and therapy in early phase clinical drug development. *Cancer Cell*. 2004;6:321-326.
11. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*. 2006;295:2727-2741.
12. Vogel VG, Costantino JP, Wickerham DL, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. *Cancer Prev Res (Phila)*. 2010;3:696-706.
13. Thompson IM, Goodman PJ, Tangen CM, et al. The influence of finasteride on the development of prostate cancer. *N Engl J Med*. 2003;349:215-224.
15. Bertagnolli MM, Eagle CJ, Zauber AG, et al. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med*. 2006;355:873-884.
16. Arber N, Eagle CJ, Spicak J, et al. Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med*. 2006;355:885-895.
17. Armstrong WB, Taylor TH, Meyskens FL. Point: Surrogate end point biomarkers are likely to be limited in their usefulness in the development of cancer chemoprevention agents against sporadic cancers. *Cancer Epidemiol Biomarkers Prev*. 2003;12:589-592.
18. Kelloff GJ, O'Shaughnessy JA, Gordon GB, et al. Counterpoint: Because some surrogate end point biomarkers measure the neoplastic process they will have high utility in the development of cancer chemopreventive agents against sporadic cancers. *Cancer Epidemiol Biomarkers Prev*. 2003;12:593-596.
19. Xiao Z, Luke BT, Izmirlian G, et al. Serum proteomic profiles suggest celecoxib-modulated targets and response predictors. *Cancer Res*. 2004;64:2904-2909.
20. Mao L, Zhang L, Lee JJ, et al. Global change of gene expression profile after 3 month administration of celecoxib, a COX-2 selected inhibitor, in bronchial epithelium of chronic smokers. *Proc Am Assoc Cancer Res*. 2005;46:1042.
21. Spira A, Beane J, Shah V, et al. Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc Natl Acad Sci U S A*. 2004;101:10143-10148.
22. Spira A, Beane J, Shah V, et al. Airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer. *Nat Med*. 2007;13:361-366.
23. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997-1006.
24. Hassanein M, Callison JC, Callaway-Lane C, et al. The state of molecular biomarkers for the early detection of lung cancer. *Cancer Prev Res (Phila)*. 2012;5:992-1006.
26. Kawaguchi H, El-Naggar AK, Papadimitrakopoulou V, et al. Podoplanin: a novel marker for oral cancer risk in patients with oral premalignancy. *J Clin Oncol*. 2008;2:354-360.
28. Makridakis N, Reichardt JK. Pharmacogenetic analysis of human steroid 5 alpha reductase type II: comparison of finasteride and dutasteroide. *J Mol Endocrinol*. 2005;34:617-623.
29. Mostaghel EA, Geng L, Holcomb I, et al. Variability in the androgen response of prostate epithelium to 5alpha-reductase inhibition: implications for prostate cancer chemoprevention. *Cancer Res*. 2010;70:1286-1295.
30. Izzo JG, Papadimitrakopoulou VA, Liu DD, et al. Cyclin D1 genotype, response to biochemoprevention, and progression rate to upper aerodigestive tract cancer. *J Natl Cancer Inst*. 2003;95:198-205.
31. Papadimitrakopoulou VA, Izzo JG, Liu DD, et al. Cyclin D1 and cancer development in laryngeal premalignacy patients. *Cancer Prev Res (Phila)*. 2009;2:14-21.
33. Tsui IFL, Rosin MP, Zhang L, et al. Multiple aberrations of chromosome 3p detected in oral premalignant lesions. *Cancer Prev Res (Phila)*. 2008;1:424-429.
35. Hildebrandt MAT, Lippman SM, Etzel CJ, et al. Genetic variants in the PI3K/PTEN/AKT/mTOR pathway predict head and neck cancer patient second primary tumor/recurrence risk and response to retinoid chemoprevention. *Clin Cancer Res*. 2012;18:3705.
36. Pierce M, Schwarz RA, Bhattar VS, et al. Accuracy of *in vivo* multimodal optical imaging for detection of oral neoplasia. *Cancer Prev Res (Phila)*. 2012;5:801-809.
37. Poh CF, MacAulay C, Zhang L, et al. Tracing the "at-risk" oral mucosa field with autofluorescence: steps toward clinical impact. *Cancer Prev Res (Phila)*. 2009;2:401-404.
38. Roblyer D, Kurachi C, Stepanek V, et al. Objective detection and delineation of oral neoplasia using autofluorescence imaging. *Cancer Prev Res (Phila)*. 2009;2:423-431.
39. Poh CF, Ng SP, Williams PM, et al. Direct fluorescence visualization of clinically occult high-risk oral premalignant disease using a simple hand-held device. *Head Neck*. 2007;29:71-76.
40. Roy HK, Gomes A, Turzhitsky V, et al. Spectroscopic microvascular blood detection from the endoscopically normal colonic mucosa: biomarker for neoplasia risk. *Gastroenterology*. 2008;4:1069-1078.
42. Hosono K, Endo H, Takahashi H, et al. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res (Phila)*. 2010;3:1077-1083.
43. Tang X, Shigematsu H, Bekele BN, et al. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res*. 2005;65:7568-7572.
44. Raso MG, Behrens C, Herynk MH, et al. Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. *Clin Cancer Res*. 2009;15:5359-5368.
45. Lippman SM, Lee JJ. Reducing the "risk" of chemoprevention: defining and targeting high risk-2005 AACR Cancer Research and Prevention Foundation Award Lecture. *Cancer Res*. 2006;66:2893-2903.
46. Guix M, Kelley MS, Reyzer ML, et al. Short course of EGF receptor tyrosine kinase inhibitor ("Tarceva") reduces tumor cell proliferation and activation in untreated operable breast cancers: a strategy for phase II trials with signaling inhibitors. *Proc Am Soc Clin Oncol*. 2005;23:194s (abstr 3008).
47. Guix M, Granja NM, Meszoely I, et al. Short preoperative treatment with erlotinib inhibits tumor cell proliferation in hormone receptor-positive breast cancers. *J Clin Oncol*. 2008;26:897-906.
48. Veronesi G, Szabo E, Decensi A, et al. Randomized phase II trial of inhaled budesonide versus placebo in high-risk individuals with CT screen-detected lung nodules. *Cancer Prev Res (Phila)*. 2011;4:34-42.
49. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. 2010;10:181-193.
50. Chan AT, Ogino S, Fuchs CS, et al. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med*. 2007;356:2131-2142.
51. Ogino S, Kirkner GJ, Nosho K, et al. Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res*. 2008;14:8221-8227.
54. Shureiqi I, Wu Y, Chen D, et al. The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. *Cancer Res*. 2005;65:11486-11492.
55. Hsi LC, Xi X, Lotan R, et al. The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells. *Cancer Res*. 2004;64:8778-8781.
56. Shureiqi I, Jiang W, Zuo X, et al. The 15-lipoxygenase-1 product 13-*S*-hydroxyoctadecadienoic acid down-regulates PPAR-delta to induce apoptosis in colorectal cancer cells. *Proc Natl Acad Sci U S A*. 2003;100:9968-9973.
57. Sherman MH, Downes M, Evans RM. Nuclear receptors as modulators of the tumor microenvironment. *Cancer Prev Res (Phila)*. 2012;5:3-10.
58. Liang ZD, Lippman SM, Wu T-T, et al. RRIG1 mediates effects of retinoic acid receptor β_2 on tumor cell growth and gene expression through binding to and inhibition of RhoA. *Cancer Res*. 2006;66:7111-7118.
59. Song S, Guan B, Men T, et al. Antitumor effect of retinoic acid receptor-beta2 associated with suppression of cyclooxygenase-2. *Cancer Prev Res (Phila)*. 2009;2:274-280.
60. Kim W-Y, Jin Q, Oh S-H, et al. Elevated epithelial insulin-like growth factor expression is a risk factor for lung cancer development. *Cancer Res*. 2009;69:7439-7448.
61. Lee H-Y, Moon HJ, Chun K-H, et al. Effects of insulin-like growth factor binding protein-3 and farnesyltransferase inhibitor SCH66336 on Akt expression and apoptosis in non-small-cell lung cancer cells. *J Natl Cancer Inst*. 2004;96:1536-1548.

62. Dolgin E. Drug companies look to biomarkers to salvage cancer target. *Nat Med*. 2012;18:1312-1313.
63. Rojas A, Liu G, Coleman I, et al. IL-6 promotes prostate tumorigenesis and progression through autocrine cross-activation of IGF-1R. *Oncogene*. 2011;30:2345-2355.
65. Lee H-Y, Oh SH, Woo J-K, et al. Chemopreventive effects of deguelin, a novel Akt inhibitor, on tobacco-induced lung tumorigenesis. *J Natl Cancer Inst*. 2005;97:1695-1699.
66. Kim WY, Oh SH, Woo JK, Hong WK, Lee HY. Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radiation-induced stabilization of hypoxia-inducible factor-1alpha. *Cancer Res*. 2009;69:1624-1632.
67. Wislez M, Spencer ML, Izzo JG, et al. Inhibition of mammalian target of rapamycin reverses alveolar epithelial neoplasia induced by oncogenic K-ras. *Cancer Res*. 2005;65:3226-3335.
68. Majumder PK, Febbo PG, Bikoff R, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med*. 2004;10:594-601.
69. Engelman JA, Cantley LC. Chemoprevention meets glucose control. *Cancer Prev Res (Phila)*. 2010;3:1049-1052.
70. Memmott RM, Mercado JR, Maier CR, et al. Metformin prevents tobacco carcinogen-induced lung tumorigenesis. *Cancer Prev Res (Phila)*. 2010;3:1066-1076.
71. Decensi A, Puntoni M, Goodwin P, et al. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev Res (Phila)*. 2010;3:1451-1461.
72. Algire C, Moiseeva O, Deschenes-Simard X, et al. Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev Res (Phila)*. 2012;5:536-543.
73. Bao B, Wang Z, Ali S, et al. Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells. *Cancer Prev Res (Phila)*. 2012;5:355-364.
74. Wislez M, Fujimoto N, Izzo JG, et al. High expression of ligands for chemokine receptor CXCR2 in alveolar epithelial neoplasia induced by oncogenic kras. *Cancer Res*. 2006;66:4198-4207.
75. Albini A, Tosetti F, Li VW, et al. Cancer prevention by targeting angiogenesis. *Nat Rev Clin Oncol*. 2012;9:498-509.
76. Sudarshan A, Cheresh DA. MicroRNA-mediated regulation of the angiogenic switch. *Curr Opin Hematol*. 2011;18:171-176.
77. Albini A, Tosetti F, Benelli R, et al. Tumor inflammatory angiogenesis and its chemoprevention. *Cancer Res*. 2005;65:10637-10641.
78. Yoshida T, Horinaka M, Sakai T."Combination-oriented molecular-targeting prevention" of cancer: a model involving the combination of TRAIL and a DR5 inducer. *Env Health Prev Med*. 2010;15:203-210.
79. Belinsky SA, Grimes MJ, Picchi MA, et al. Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer Res*. 2011;71:454-462.
80. Lippman SM, Gibson N, Subbaramaiah K, et al. Combined targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways. *Clin Cancer Res*. 2005;11:6097-6099.
81. Subbaramaiah K, Howe LR, Port ER, et al. HER-2/neu status is a determinant of mammary aromatase activity in vivo: evidence for a cyclooxygenase-2-dependent mechanism. *Cancer Res*. 2006;66:5504-5511.
82. Bruzzese F, Leone A, Rocco M, et al. HDAC inhibitor vorinostat enhances the antitumor effect of gefitinib in squamous cell carcinoma of head and neck by modulating ErbB receptor expression and reverting EMT. *J Cell Physiol*. 2011;226:2378-2390.
84. Garnis C, Chari R, Buys TP, et al. Genomic imbalances in precancerous tissues signal oral cancer risk. *Mol Cancer*. 2009;8:50.
85. Zhang L, Poh CF, Williams M, et al. Loss of heterozygosity (LOH) profiles—validated risk predictors for progression to oral cancer. *Cancer Prev Res (Phila)*. 2012;5:1081-1089.
86. Pena Murillo C, Huang X, Hills A, et al. The utility of molecular diagnostics to predict recurrence of head and neck carcinoma. *Br J Cancer*. 2012;107:1138-1143.
87. Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. *Cancer Cell*. 2004;5:311-316.
90. Guillaud M, Zhang L, Poh C, et al. Potential use of quantitative tissue phenotype to predict malignant risk for oral premalignant lesions. *Cancer Res*. 2008;68:3099-3107.
91. Saintigny P, Zhang L, Fan YH, et al. Gene expression profiling predicts the development of oral cancer. *Cancer Prev Res (Phila)*. 2011;4:218-229.
92. Maley CC, Galipeau PC, Li X, et al. The combination of genetic instability and clonal expansion predicts progression to esophageal adenocarcinoma. *Cancer Res*. 2004;64:7629-7633.
93. Maley CC, Galipeau PC, Finley JC, et al. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. *Nat Genet*. 2006;38:468-473.
94. Li X, Galipeau PC, Sanchez CA, et al. Single nucleotide polymorphism-based genome-wide chromosome copy change, loss of heterozygosity, and aneuploidy in Barrett's esophagus neoplastic progression. *Cancer Prev Res (Phila)*. 2008;1:413-423.
95. Yang L, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration is esophagitis and Barrett esophagus. *Clin Cancer Res*. 2012;18:2138-2144.
98. Anothaisintawee T, Teerawattananon Y, Wiratkapun C, et al. Risk prediction models of breast cancer: A systematic review of model performances. *Breast Cancer Res Treat*. 2012;133:1-10.
100. Gail MH, Mai PL. Comparing breast cancer risk assessment models. *J Natl Cancer Inst*. 2010;102:665-668.
101. Ferlay J, Shin H-R, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.
102. D'Amelio AM, Cassidy A, Asomaning K, et al. Comparison of discriminatory power and accuracy of three lung cancer risk models. *Br J Cancer*. 2010;103:423-429.
103. Foy M, Spitz M, Kimmel M, et al. A smoking-based carcinogenesis model for lung cancer risk prediction. *Int J Cancer*. 2011;129:1907-1913.
104. Tammemagi CM, Pinsky PF, Caporaso NE, et al. Lung cancer risk prediction: Prostate, lung, colorectal and ovarian cancer screening trial models and validation. *J Natl Cancer Inst*. 2011;103:1058-1068.
107. Khuri FR, Lee JJ, Lippman SM, et al. Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients. *J Natl Cancer Inst*. 2006;98:441-450.
111. Lippman SM, Klein EA, Goodman PJ, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. 2009;301:39-51.
112. Klein EA, Thompson Jr IM, Tangen CM, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. 2011;306:1549-1556.
114. Qiao YL, Dawsey SM, Kamangar F, et al. Total and cancer mortality after supplementation with vitamins and minerals: follow-up of the Linxian General Population Nutrition Intervention Trial. *J Natl Cancer Inst*. 2009;101:507-518.
115. Overholt BF, Lightdale CJ, Wang KK, et al. Photodynamic therapy with porfimer sodium for ablation of high-grade dysplasia in Barrett's esophagus: international, partially blinded, randomized phase III trial. *Gastrointest Endosc*. 2005;62:488-498.
116. Overholt BF, Wang KK, Burdick JS, et al. Five-year efficacy and safety of photodynamic therapy with Photofrin in Barrett's high-grade dysplasia. *Gastrointest Endosc*. 2007;66:460-468.
117. Sandler RS, Halabi S, Baron JA, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med*. 2003;348:883-890.
118. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med*. 2003;348:891-899.
119. Gann PH, Manson JE, Glynn RJ, et al. Low-dose aspirin and incidence of colorectal tumors in a randomized trial. *J Natl Cancer Inst*. 1993;85:1220-1224.
120. Burn J, Bishop DT, Chapman PD, et al. A randomized placebo-controlled prevention trial of aspirin and/or resistant starch in young people with familial adenomatous polyposis. *Cancer Prev Res (Phila)*. 2011;4:655-665.
121. Cole BF, Logan RF, Halabi S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. *J Natl Cancer Inst*. 2009;101:256066.
123. Burn J, Gerdes AM, Macrae F, et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet*. 2011;378:2081-2087.

124. Rothwell PM, Price JF, Fowkes FGR, et al. Short-term effects of daily aspirin cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet*. 2012;379:1602-1612.
126. Pierce JP, Natarajan L, Caan BJ, et al. Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: the Women's Healthy Eating and Living (WHEL) randomized trial. *JAMA*. 2007;289:289-298.
127. Chen T, Yan F, Qian J, et al. Randomized phase II trial of lyophilized strawberries in patients with dysplastic precancerous lesions of the esophagus. *Cancer Prev Res (Phila)*. 2012;5:41-50.
128. Kensler TW, Ng D, Carmelia SG, et al. Modulation of the metabolism of airborne pollutants by glucoraphanin-rich and sulforaphane-rich broccoli sprout beverages in Qidong, China. *Carcinogenesis*. 2012;33:101-107.
129. Tsao AS, Liu D, Martin J, et al. Phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions. *Cancer Prev Res (Phila)*. 2009;2:931-942.
131. ATAC Trialists' Group. Results of ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet*. 2005;365:60-62.
132. Goss PE, Ingle JN, Ales-Martinez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med*. 2011;364:2381-2391.
134. Lu C, Speers C, Zhang Y, et al. Effect of epidermal growth factor receptor inhibitor on development of estrogen receptor-negative mammary tumors. *J Natl Cancer Inst*. 2003;95:1825-1833.
135. Howe LR, Brown PH. Targeting the HER/EGFR/ErbB family to prevent breast cancer. *Cancer Prev Res (Phila)*. 2011;4:1149-1157.
136. Untch M, Loibl S, Bischoff J, et al. Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomized Phase III trial. *Lancet Oncol*. 2012;13:135-144.
137. Chang JC, Mayer IA, Forero-Torres A, et al. TBCRC 006: a multicenter phase II study of neoadjuvant lapatinib and trastuzumab in patients with HER2-overexpressing breast cancer. *J Clin Oncol*. 2011;29:166-173.
138. Blackwell KL, Burstein HJ, Storniolo AM, et al. Randomized study of lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic inflammatory breast cancer. *J Clin Oncol*. 2010;28:1124-1130.
139. Andriole GL, Bostwick DG, Brawley OW, et al. Chemoprevention of prostate cancer in men at high risk: rationale and design of the reduction by dutasteride of prostate cancer events (REDUCE) trial. *J Urol*. 2004;172:1314-1317.
140. Andriole GL, Bostwick DG, Brawley OW, et al. Effect of dutasteride on the risk of prostate cancer. *N Engl J Med*. 2010;362:1192-1202.
141. Walsh PC. Chemoprevention of prostate cancer: comment on effect of dutasteride on the risk of prostate cancer. *N Engl J Med*. 2010;362:1237-1238.
142. Bresalier RS, Sandler RS, Quan H, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med*. 2005;352:1092-1102.
143. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med*. 2005;352:1071-1080.
144. Federal Register 2012;77:34052.
145. Chan AT, Sima CS, Zauber AG, et al. C-reactive protein and risk of colorectal adenoma according to celecoxib treatment. *Cancer Prev Res (Phila)*. 2011;4:1172-1180.
146. Mao JT, Roth MD, Fishbein MC, et al. Lung cancer chemoprevention with celecoxib in former smokers. *Cancer Prev Res (Phila)*. 2011;4:984-993.
147. Keith RL, Blatchford PJ, Kittelson J, et al. Oral iloprost improves endobronchial dysplasia in former smokers. *Cancer Prev Res (Phila)*. 2011;4:793-802.
150. Chang MH, You SL, Chen CJ, et al. Taiwan Hepatoma Study Group. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccines: a 20-year follow-up study. *J Natl Cancer Inst*. 2009;101:1348-1355.
151. Lowy DR, Shiller JT. Reducing HPV-associated cancer globally. *Cancer Prev Res (Phila)*. 2012;5:18-23.
152. Pierce Campbell CM, Menezes LJ, Paskett ED, et al. Prevention of invasive cervical cancer in the United States: past, present, and future. *Cancer Epidemiol Biomarkers Prev*. 2102;21:1402-1408.
153. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009-2010. *JAMA*. 2012;307:693-703.
154. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011;29:4294-4301.
155. Settle K, Posner MR, Schumaker LM, et al. Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal cancer patients. *Cancer Prev Res (Phila)*. 2009;2:776-781.
156. Greenberg ER, Anderson GL, Morgan DR, et al. 14-day triple, 5-day concomitant, and 10-day sequential therapies for *Helicobacter pylori* infection in seven Latin American sites: a randomized trial. *Lancet*. 2011;378:507-514.
157. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet*. 2012;13:607-615.
158. Finn OJ. Premalignant lesions as targets for cancer vaccines. *J Exp Med*. 2003;198:1623-1626.
159. Dhodapkar MV. Harnessing host immune responses to preneoplasia: promise and challenges. *Cancer Immunol Immunother*. 2005;65:409-413.
160. Spisek R, Dhodapkar MV. Immunoprevention of cancer. *Hematol Oncol Clin North Am*. 2006;20:735-750.
161. Weiner LM, Surana R, Murray J. Vaccine prevention of cancer: can endogenous antigens be targeted? *Cancer Prev Res (Phila)*. 2010;3:410-415.
163. Kelloff GJ, Lippman SM, Dannenberg AJ, et al. Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer-a plan to move forward. *Clin Cancer Res*. 2006;12:3661-3697.
164. Lippman SM, Heymach JV. The convergent development of molecular-targeted drugs for cancer treatment and prevention. *Clin Cancer Res*. 2007;13:4035-4041.
165. Herbst RS, Lippman SM. Molecular signatures of lung cancer—toward personalized therapy. *N Engl J Med*. 2007;356:76-78.
166. Reid BJ. Cancer risk assessment and cancer prevention: promises and challenges. *Cancer Prev Res (Phila)*. 2008;1:229-232.

References

1. Lippman SM, Hong WK. Cancer prevention science and practice. *Cancer Res*. 2002;62:5119-5125.
2. O'Shaughnessy JA, Kelloff GJ, Gordon GB, et al. Treatment and prevention of intraepithelial neoplasia: an important target for accelerated new agent development. *Clin Cancer Res*. 2002;8:314-346.
3. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science*. 1997;278:1073-1077.
4. Lotan R, Xu XC, Lippman SM, et al. Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med*. 1995;332:1405-1410.
5. Mao L, Lee JS, Fan YH, et al. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. *Nat Med*. 1996;2:682-685.
6. Hong WK, Endicott J, Itri LM, et al. 13-cis retinoic acid in the treatment of oral leukoplakia. *N Engl J Med*. 1986;315:1501-1505.
7. Lippman SM, Batsakis JG, Toth BB, et al. Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis. *N Engl J Med*. 1993;328:15-20.
8. Abbruzzese JL, Lippman SM. The convergence of cancer prevention and therapy in early phase clinical drug development. *Cancer Cell*. 2004;6:321-326.
9. Jang SJ, Chiba I, Hirai A, et al. Multiple oral squamous epithelial lesions: are they genetically related? *Oncogene*. 2001;20:2235-2242.
10. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst*. 1998;90:1371-1388.
11. Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA*. 2006;295:2727-2741.
12. Vogel VG, Costantino JP, Wickerham DL, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial: preventing breast cancer. *Cancer Prev Res (Phila)*. 2010;3:696-706.
13. Thompson IM, Goodman PJ, Tangen CM, et al. The influence of finasteride on the development of prostate cancer. *N Engl J Med*. 2003;349:215-224.
14. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N Engl J Med*. 2000;342:1946-1952.
15. Bertagnolli MM, Eagle CJ, Zauber AG, et al. Celecoxib for the prevention of sporadic colorectal adenomas. *N Engl J Med*. 2006;355:873-884.
16. Arber N, Eagle CJ, Spicak J, et al. Celecoxib for the prevention of colorectal adenomatous polyps. *N Engl J Med*. 2006;355:885-895.
17. Armstrong WB, Taylor TH, Meyskens FL. Point: Surrogate end point biomarkers are likely to be limited in their usefulness in the development of cancer chemoprevention agents against sporadic cancers. *Cancer Epidemiol Biomarkers Prev*. 2003;12:589-592.
18. Kelloff GJ, O'Shaughnessy JA, Gordon GB, et al. Counterpoint: Because some surrogate end point biomarkers measure the neoplastic process they will have high utility in the development of cancer chemopreventive agents against sporadic cancers. *Cancer Epidemiol Biomarkers Prev*. 2003;12:593-596.
19. Xiao Z, Luke BT, Izmirlian G, et al. Serum proteomic profiles suggest celecoxib-modulated targets and response predictors. *Cancer Res*. 2004;64:2904-2909.
20. Mao L, Zhang L, Lee JJ, et al. Global change of gene expression profile after 3 month administration of celecoxib, a COX-2 selected inhibitor, in bronchial epithelium of chronic smokers. *Proc Am Assoc Cancer Res*. 2005;46:1042.
21. Spira A, Beane J, Shah V, et al. Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc Natl Acad Sci U S A*. 2004;101:10143-10148.
22. Spira A, Beane J, Shah V, et al. Airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer. *Nat Med*. 2007;13:361-366.
23. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997-1006.
24. Hassanein M, Callison JC, Callaway-Lane C, et al. The state of molecular biomarkers for the early detection of lung cancer. *Cancer Prev Res (Phila)*. 2012;5:992-1006.
25. Mao L, El-Naggar AK, Papadimitrakopoulou V, et al. Phenotype and genotype of advanced premalignant head and neck lesions after chemopreventive therapy. *J Natl Cancer Inst*. 1998;90:1545-1551.
26. Kawaguchi H, El-Naggar AK, Papadimitrakopoulou V, et al. Podoplanin: a novel marker for oral cancer risk in patients with oral premalignancy. *J Clin Oncol*. 2008;2:354-360.
27. King MC, Wieand S, Hale K, et al. Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial. *JAMA*. 2001;286:2251-2256.
28. Makridakis N, Reichardt JK. Pharmacogenetic analysis of human steroid 5 alpha reductase type II: comparison of finasteride and dutasteroide. *J Mol Endocrinol*. 2005;34:617-623.
29. Mostaghel EA, Geng L, Holcomb I, et al. Variability in the androgen response of prostate epithelium to 5alpha-reductase inhibition: implications for prostate cancer chemoprevention. *Cancer Res*. 2010;70:1286-1295.
30. Izzo JG, Papadimitrakopoulou VA, Liu DD, et al. Cyclin D1 genotype, response to biochemoprevention, and progression rate to upper aerodigestive tract cancer. *J Natl Cancer Inst*. 2003;95:198-205.
31. Papadimitrakopoulou VA, Izzo JG, Liu DD, et al. Cyclin D1 and cancer development in laryngeal premalignacy patients. *Cancer Prev Res (Phila)*. 2009;2:14-21.
32. Rosin MP, Lam WL, Poh C, et al. 3p14 and 9p21 loss is a simple tool for predicting second oral malignancy at previously treated oral cancer sites. *Cancer Res*. 2002;62:6447-6450.
33. Tsui IFL, Rosin MP, Zhang L, et al. Multiple aberrations of chromosome 3p detected in oral premalignant lesions. *Cancer Prev Res (Phila)*. 2008;1:424-429.
34. Khuri FR, Rodriguez M, Lee JJ, et al. Comprehensive analysis of clinical and molecular determinants of second events in the randomized retinoid head and neck second primary tumor prevention trial. *Proc Am Assoc Cancer Res*. 2002;43 (abstr 2553).
35. Hildebrandt MAT, Lippman SM, Etzel CJ, et al. Genetic variants in the PI3K/PTEN/AKT/mTOR pathway predict head and neck cancer patient second primary tumor/recurrence risk and response to retinoid chemoprevention. *Clin Cancer Res*. 2012;18:3705.
36. Pierce M, Schwarz RA, Bhattar VS, et al. Accuracy of *in vivo* multimodal optical imaging for detection of oral neoplasia. *Cancer Prev Res (Phila)*. 2012;5:801-809.
37. Poh CF, MacAulay C, Zhang L, et al. Tracing the "at-risk" oral mucosa field with autofluorescence: steps toward clinical impact. *Cancer Prev Res (Phila)*. 2009;2:401-404.
38. Roblyer D, Kurachi C, Stepanek V, et al. Objective detection and delineation of oral neoplasia using autofluorescence imaging. *Cancer Prev Res (Phila)*. 2009;2:423-431.
39. Poh CF, Ng SP, Williams PM, et al. Direct fluorescence visualization of clinically occult high-risk oral premalignant disease using a simple hand-held device. *Head Neck*. 2007;29:71-76.
40. Roy HK, Gomes A, Turzhitsky V, et al. Spectroscopic microvascular blood detection from the endoscopically normal colonic mucosa: biomarker for neoplasia risk. *Gastroenterology*. 2008;4:1069-1078.
41. Takayama T, Katsuki S, Takahashi Y, et al. Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med*. 1998;339:1277-1284.
42. Hosono K, Endo H, Takahashi H, et al. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res (Phila)*. 2010;3:1077-1083.
43. Tang X, Shigematsu H, Bekele BN, et al. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res*. 2005;65:7568-7572.
44. Raso MG, Behrens C, Herynk MH, et al. Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. *Clin Cancer Res*. 2009;15:5359-5368.
45. Lippman SM, Lee JJ. Reducing the "risk" of chemoprevention: defining and targeting high risk-2005 AACR Cancer Research and Prevention Foundation Award Lecture. *Cancer Res*. 2006;66:2893-2903.

46. Guix M, Kelley MS, Reyzer ML, et al. Short course of EGF receptor tyrosine kinase inhibitor ("Tarceva") reduces tumor cell proliferation and activation in untreated operable breast cancers: a strategy for phase II trials with signaling inhibitors. *Proc Am Soc Clin Oncol*. 2005;23:194s (abstr 3008).
47. Guix M, Granja NM, Meszoely I, et al. Short preoperative treatment with erlotinib inhibits tumor cell proliferation in hormone receptor-positive breast cancers. *J Clin Oncol*. 2008;26:897-906.
48. Veronesi G, Szabo E, Decensi A, et al. Randomized phase II trial of inhaled budesonide versus placebo in high-risk individuals with CT screen-detected lung nodules. *Cancer Prev Res (Phila)*. 2011;4:34-42.
49. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. 2010;10:181-193.
50. Chan AT, Ogino S, Fuchs CS, et al. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med*. 2007;356:2131-2142.
51. Ogino S, Kirkner GJ, Nosho K, et al. Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res*. 2008;14:8221-8227.
52. Eberhart CE, Coffey RJ, Radhika FM, et al. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*. 1994;107:1183-1188.
53. Shureiqi I, Chen D, Lee JJ, et al. 15-LOX-1: a novel molecular target of nonsteroidal anti-inflammatory drug-induced apoptosis in colorectal cancer cells. *J Natl Cancer Inst*. 2000;92:1136-1142.
54. Shureiqi I, Wu Y, Chen D, et al. The critical role of 15-lipoxygenase-1 in colorectal epithelial cell terminal differentiation and tumorigenesis. *Cancer Res*. 2005;65:11486-11492.
55. Hsi LC, Xi X, Lotan R, et al. The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) induces apoptosis via induction of 15-lipoxygenase-1 in colorectal cancer cells. *Cancer Res*. 2004;64:8778-8781.
56. Shureiqi I, Jiang W, Zuo X, et al. The 15-lipoxygenase-1 product 13-*S*-hydroxyoctadecadienoic acid down-regulates PPAR-delta to induce apoptosis in colorectal cancer cells. *Proc Natl Acad Sci U S A*. 2003;100:9968-9973.
57. Sherman MH, Downes M, Evans RM. Nuclear receptors as modulators of the tumor microenvironment. *Cancer Prev Res (Phila)*. 2012;5:3-10.
58. Liang ZD, Lippman SM, Wu T-T, et al. RRIG1 mediates effects of retinoic acid receptor β_2 on tumor cell growth and gene expression through binding to and inhibition of RhoA. *Cancer Res*. 2006;66:7111-7118.
59. Song S, Guan B, Men T, et al. Antitumor effect of retinoic acid receptor-beta2 associated with suppression of cyclooxygenase-2. *Cancer Prev Res (Phila)*. 2009;2:274-280.
60. Kim W-Y, Jin Q, Oh S-H, et al. Elevated epithelial insulin-like growth factor expression is a risk factor for lung cancer development. *Cancer Res*. 2009;69:7439-7448.
61. Lee H-Y, Moon HJ, Chun K-H, et al. Effects of insulin-like growth factor binding protein-3 and farnesyltransferase inhibitor SCH66336 on Akt expression and apoptosis in non-small-cell lung cancer cells. *J Natl Cancer Inst*. 2004;96:1536-1548.
62. Dolgin E. Drug companies look to biomarkers to salvage cancer target. *Nat Med*. 2012;18:1312-1313.
63. Rojas A, Liu G, Coleman I, et al. IL-6 promotes prostate tumorigenesis and progression through autocrine cross-activation of IGF-1R. *Oncogene*. 2011;30:2345-2355.
64. Jyonouchi H, Sun S, Iijima K, et al. Effects of anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene on human small airway epithelial cells and the protective effects of myo-inositol. *Carcinogenesis*. 1999;20:139-145.
65. Lee H-Y, Oh SH, Woo J-K, et al. Chemopreventive effects of deguelin, a novel Akt inhibitor, on tobacco-induced lung tumorigenesis. *J Natl Cancer Inst*. 2005;97:1695-1699.
66. Kim WY, Oh SH, Woo JK, Hong WK, Lee HY. Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radiation-induced stabilization of hypoxia-inducible factor-1alpha. *Cancer Res*. 2009;69:1624-1632.
67. Wislez M, Spencer ML, Izzo JG, et al. Inhibition of mammalian target of rapamycin reverses alveolar epithelial neoplasia induced by oncogenic K-ras. *Cancer Res*. 2005;65:3226-3335.
68. Majumder PK, Febbo PG, Bikoff R, et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med*. 2004;10:594-601.
69. Engelman JA, Cantley LC. Chemoprevention meets glucose control. *Cancer Prev Res (Phila)*. 2010;3:1049-1052.
70. Memmott RM, Mercado JR, Maier CR, et al. Metformin prevents tobacco carcinogen-induced lung tumorigenesis. *Cancer Prev Res (Phila)*. 2010;3:1066-1076.
71. Decensi A, Puntoni M, Goodwin P, et al. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev Res (Phila)*. 2010;3:1451-1461.
72. Algire C, Moiseeva O, Deschenes-Simard X, et al. Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev Res (Phila)*. 2012;5:536-543.
73. Bao B, Wang Z, Ali S, et al. Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells. *Cancer Prev Res (Phila)*. 2012;5:355-364.
74. Wislez M, Fujimoto N, Izzo JG, et al. High expression of ligands for chemokine receptor CXCR2 in alveolar epithelial neoplasia induced by oncogenic kras. *Cancer Res*. 2006;66:4198-4207.
75. Albini A, Tosetti F, Li VW, et al. Cancer prevention by targeting angiogenesis. *Nat Rev Clin Oncol*. 2012;9:498-509.
76. Sudarshan A, Cheresh DA. MicroRNA-mediated regulation of the angiogenic switch. *Curr Opin Hematol*. 2011;18:171-176.
77. Albini A, Tosetti F, Benelli R, et al. Tumor inflammatory angiogenesis and its chemoprevention. *Cancer Res*. 2005;65:10637-10641.
78. Yoshida T, Horinaka M, Sakai T. "Combination-oriented molecular-targeting prevention" of cancer: a model involving the combination of TRAIL and a DR5 inducer. *Env Health Prev Med*. 2010;15:203-210.
79. Belinsky SA, Grimes MJ, Picchi MA, et al. Combination therapy with vidaza and entinostat suppresses tumor growth and reprograms the epigenome in an orthotopic lung cancer model. *Cancer Res*. 2011;71:454-462.
80. Lippman SM, Gibson N, Subbaramaiah K, et al. Combined targeting of the epidermal growth factor receptor and cyclooxygenase-2 pathways. *Clin Cancer Res*. 2005;11:6097-6099.
81. Subbaramaiah K, Howe LR, Port ER, et al. HER-2/neu status is a determinant of mammary aromatase activity in vivo: evidence for a cyclooxygenase-2-dependent mechanism. *Cancer Res*. 2006;66:5504-5511.
82. Bruzzese F, Leone A, Rocco M, et al. HDAC inhibitor vorinostat enhances the antitumor effect of gefitinib in squamous cell carcinoma of head and neck by modulating ErbB receptor expression and reverting EMT. *J Cell Physiol*. 2011;226:2378-2390.
83. Rosin MP, Cheng X, Poh CF, et al. Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. *Clin Cancer Res*. 2000;6:357-362.
84. Garnis C, Chari R, Buys TP, et al. Genomic imbalances in precancerous tissues signal oral cancer risk. *Mol Cancer*. 2009;8:50.
85. Zhang L, Poh CF, Williams M, et al. Loss of heterozygosity (LOH) profiles—validated risk predictors for progression to oral cancer. *Cancer Prev Res (Phila)*. 2012;5:1081-1089.
86. Pena Murillo C, Huang X, Hills A, et al. The utility of molecular diagnostics to predict recurrence of head and neck carcinoma. *Br J Cancer*. 2012;107:1138-1143.
87. Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. *Cancer Cell*. 2004;5:311-316.
88. Lippman SM, Hong WK. Molecular markers of the risk of oral cancer. *N Engl J Med*. 2001;344:1323-1326.
89. Lee JJ, Hong WK, Hittelman WN, et al. Predicting cancer development in oral leukoplakia: ten years of translational research. *Clin Cancer Res*. 2000;6:1702-1710.
90. Guillaud M, Zhang L, Poh C, et al. Potential use of quantitative tissue phenotype to predict malignant risk for oral premalignant lesions. *Cancer Res*. 2008;68:3099-3107.
91. Saintigny P, Zhang L, Fan YH, et al. Gene expression profiling predicts the development of oral cancer. *Cancer Prev Res (Phila)*. 2011;4:218-229.
92. Maley CC, Galipeau PC, Li X, et al. The combination of genetic instability and clonal expansion predicts progression to esophageal adenocarcinoma. *Cancer Res*. 2004;64:7629-7633.

93. Maley CC, Galipeau PC, Finley JC, et al. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. *Nat Genet*. 2006;38:468-473.
94. Li X, Galipeau PC, Sanchez CA, et al. Single nucleotide polymorphism-based genome-wide chromosome copy change, loss of heterozygosity, and aneuploidy in Barrett's esophagus neoplastic progression. *Cancer Prev Res (Phila)*. 2008;1:413-423.
95. Yang L, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration is esophagitis and Barrett esophagus. *Clin Cancer Res*. 2012;18:2138-2144.
96. Gail MH, Brinton LA, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. *J Natl Cancer Inst*. 1989;81:1879-1886.
97. Rosner B. Colditz GA[1996] Nurses' health study: log-incidence mathematical model of breast cancer incidence. *J Natl Cancer Inst*. 1996;88:359-364.
98. Anothaisintawee T, Teerawattananon Y, Wiratkapun C, et al. Risk prediction models of breast cancer: A systematic review of model performances. *Breast Cancer Res Treat*. 2012;133:1-10.
99. Gail MH, Costantino JP, Bryant J, et al. Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer. *J Natl Cancer Inst*. 1991;21:1829-1846.
100. Gail MH, Mai PL. Comparing breast cancer risk assessment models. *J Natl Cancer Inst*. 2010;102:665-668.
101. Ferlay J, Shin H-R, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*. 2010;127:2893-2917.
102. D'Amelio AM, Cassidy A, Asomaning K, et al. Comparison of discriminatory power and accuracy of three lung cancer risk models. *Br J Cancer*. 2010;103:423-429.
103. Foy M, Spitz M, Kimmel M, et al. A smoking-based carcinogenesis model for lung cancer risk prediction. *Int J Cancer*. 2011;129:1907-1913.
104. Tammemagi CM, Pinsky PF, Caporaso NE, et al. Lung cancer risk prediction: Prostate, lung, colorectal and ovarian cancer screening trial models and validation. *J Natl Cancer Inst*. 2011;103:1058-1068.
105. Hong WK, Lippman SM, Itri LM, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med*. 1990;323:795-801.
106. Vokes EE, Weichselbaum RR, Lippman SM, et al. Head and neck cancer. *N Engl J Med*. 1993;328:184-194.
107. Khuri FR, Lee JJ, Lippman SM, et al. Randomized phase III trial of low-dose isotretinoin for prevention of second primary tumors in stage I and II head and neck cancer patients. *J Natl Cancer Inst*. 2006;98:441-450.
108. Lippman SM, Lee JJ, Karp DD, et al. Randomized phase III intergroup trial of isotretinoin to prevent second primary tumors in stage I non-small cell lung cancer. *J Natl Cancer Inst*. 2001;93:605-618.
109. The Alpha-Tocopherol. Beta-Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med*. 1994;330:1029-1035.
110. Ommen GS, Goodman GE, Thornquist MD, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med*. 1996;334:1150-1155.
111. Lippman SM, Klein EA, Goodman PJ, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. 2009;301:39-51.
112. Klein EA, Thompson Jr IM, Tangen CM, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. 2011;306:1549-1556.
113. Blot WJ, Li JY, Taylor PR, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst*. 1993;85:1483-1492.
114. Qiao YL, Dawsey SM, Kamangar F, et al. Total and cancer mortality after supplementation with vitamins and minerals: follow-up of the Linxian General Population Nutrition Intervention Trial. *J Natl Cancer Inst*. 2009;101:507-518.
115. Overholt BF, Lightdale CJ, Wang KK, et al. Photodynamic therapy with porfimer sodium for ablation of high-grade dysplasia in Barrett's esophagus: international, partially blinded, randomized phase III trial. *Gastrointest Endosc*. 2005;62:488-498.
116. Overholt BF, Wang KK, Burdick JS, et al. Five-year efficacy and safety of photodynamic therapy with Photofrin in Barrett's high-grade dysplasia. *Gastrointest Endosc*. 2007;66:460-468.
117. Sandler RS, Halabi S, Baron JA, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N Engl J Med*. 2003;348:883-890.
118. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. *N Engl J Med*. 2003;348:891-899.
119. Gann PH, Manson JE, Glynn RJ, et al. Low-dose aspirin and incidence of colorectal tumors in a randomized trial. *J Natl Cancer Inst*. 1993;85:1220-1224.
120. Burn J, Bishop DT, Chapman PD, et al. A randomized placebo-controlled prevention trial of aspirin and/or resistant starch in young people with familial adenomatous polyposis. *Cancer Prev Res (Phila)*. 2011;4:655-665.
121. Cole BF, Logan RF, Halabi S, et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. *J Natl Cancer Inst*. 2009;101:256066.
122. Baron JA, Beach M, Mandel JS, et al. Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N Engl J Med*. 1999;340:101-107.
123. Burn J, Gerdes AM, Macrae F, et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet*. 2011;378:2081-2087.
124. Rothwell PM, Price JF, Fowkes FGR, et al. Short-term effects of daily aspirin cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet*. 2012;379:1602-1612.
125. Schatzkin A, Lanza E, Corle D, et al. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group. *N Engl J Med*. 2000;342:1149-1155.
126. Pierce JP, Natarajan L, Caan BJ, et al. Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: the Women's Healthy Eating and Living (WHEL) randomized trial. *JAMA*. 2007;289:289-298.
127. Chen T, Yan F, Qian J, et al. Randomized phase II trial of lyophilized strawberries in patients with dysplastic precancerous lesions of the esophagus. *Cancer Prev Res (Phila)*. 2012;5:41-50.
128. Kensler TW, Ng D, Carmelia SG, et al. Modulation of the metabolism of airborne pollutants by glucoraphanin-rich and sulforaphane-rich broccoli sprout beverages in Qidong, China. *Carcinogenesis*. 2012;33:101-107.
129. Tsao AS, Liu D, Martin J, et al. Phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions. *Cancer Prev Res (Phila)*. 2009;2:931-942.
130. Cuzick J, Forbes J, Edwards R, et al. First results from the International Breast Cancer Intervention Study (IBIS-I): a randomized prevention trial. *Lancet*. 2002;360:817-824.
131. ATAC Trialists' Group. Results of ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. *Lancet*. 2005;365:60-62.
132. Goss PE, Ingle JN, Ales-Martinez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med*. 2011;364:2381-2391.
133. Lippman SM, Brown PH. Tamoxifen prevention of breast cancer: an instance of the fingerpost. *J Natl Cancer Inst*. 1999;91:1809-1819.
134. Lu C, Speers C, Zhang Y, et al. Effect of epidermal growth factor receptor inhibitor on development of estrogen receptor-negative mammary tumors. *J Natl Cancer Inst*. 2003;95:1825-1833.
135. Howe LR, Brown PH. Targeting the HER/EGFR/ErbB family to prevent breast cancer. *Cancer Prev Res (Phila)*. 2011;4:1149-1157.
136. Untch M, Loibl S, Bischoff J, et al. Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomized Phase III trial. *Lancet Oncol*. 2012;13:135-144.
137. Chang JC, Mayer IA, Forero-Torres A, et al. TBCRC 006: a multicenter phase II study of neoadjuvant lapatinib and trastuzumab in patients with HER2-overexpressing breast cancer. *J Clin Oncol*. 2011;29:166-173.
138. Blackwell KL, Burstein HJ, Storniolo AM, et al. Randomized study of lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic inflammatory breast cancer. *J Clin Oncol*. 2010;28:1124-1130.

139. Andriole GL, Bostwick DG, Brawley OW, et al. Chemoprevention of prostate cancer in men at high risk: rationale and design of the reduction by dutasteride of prostate cancer events (REDUCE) trial. *J Urol*. 2004;172:1314-1317.
140. Andriole GL, Bostwick DG, Brawley OW, et al. Effect of dutasteride on the risk of prostate cancer. *N Engl J Med*. 2010;362:1192-1202.
141. Walsh PC. Chemoprevention of prostate cancer: comment on effect of dutasteride on the risk of prostate cancer. *N Engl J Med*. 2010;362:1237-1238.
142. Bresalier RS, Sandler RS, Quan H, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med*. 2005;352:1092-1102.
143. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med*. 2005;352:1071-1080.
144. Federal Register 2012;77:34052.
145. Chan AT, Sima CS, Zauber AG, et al. C-reactive protein and risk of colorectal adenoma according to celecoxib treatment. *Cancer Prev Res (Phila)*. 2011;4:1172-1180.
146. Mao JT, Roth MD, Fishbein MC, et al. Lung cancer chemoprevention with celecoxib in former smokers. *Cancer Prev Res (Phila)*. 2011;4:984-993.
147. Keith RL, Blatchford PJ, Kittelson J, et al. Oral iloprost improves endobronchial dysplasia in former smokers. *Cancer Prev Res (Phila)*. 2011;4:793-802.
148. Chang MH, Chen CJ, Lai MS, et al. Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med*. 1997;336:1855-1859.
149. Chang MH, Shau WY, Chen CJ, et al. Hepatitis B vaccination and hepatocellular carcinoma rates in boys and girls. *JAMA*. 2000;284:3040-3042.
150. Chang MH, You SL, Chen CJ, et al. Taiwan Hepatoma Study Group. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccines: a 20-year follow-up study. *J Natl Cancer Inst*. 2009;101:1348-1355.
151. Lowy DR, Shiller JT. Reducing HPV-associated cancer globally. *Cancer Prev Res (Phila)*. 2012;5:18-23.
152. Pierce Campbell CM, Menezes LJ, Paskett ED, et al. Prevention of invasive cervical cancer in the United States: past, present, and future. *Cancer Epidemiol Biomarkers Prev*. 2102;21:1402-1408.
153. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009-2010. *JAMA*. 2012;307:693-703.
154. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol*. 2011;29:4294-4301.
155. Settle K, Posner MR, Schumaker LM, et al. Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal cancer patients. *Cancer Prev Res (Phila)*. 2009;2:776-781.
156. Greenberg ER, Anderson GL, Morgan DR, et al. 14-day triple, 5-day concomitant, and 10-day sequential therapies for *Helicobacter pylori* infection in seven Latin American sites: a randomized trial. *Lancet*. 2011;378:507-514.
157. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet*. 2012;13:607-615.
158. Finn OJ. Premalignant lesions as targets for cancer vaccines. *J Exp Med*. 2003;198:1623-1626.
159. Dhodapkar MV. Harnessing host immune responses to preneoplasia: promise and challenges. *Cancer Immunol Immunother*. 2005;65:409-413.
160. Spisek R, Dhodapkar MV. Immunoprevention of cancer. *Hematol Oncol Clin North Am*. 2006;20:735-750.
161. Weiner LM, Surana R, Murray J. Vaccine prevention of cancer: can endogenous antigens be targeted? *Cancer Prev Res (Phila)*. 2010;3:410-415.
162. Lippman SM, Hong WK. Cancer prevention by delay. *Clin Cancer Res*. 2002;8:305-313.
163. Kelloff GJ, Lippman SM, Dannenberg AJ, et al. Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer-a plan to move forward. *Clin Cancer Res*. 2006;12:3661-3697.
164. Lippman SM, Heymach JV. The convergent development of molecular-targeted drugs for cancer treatment and prevention. *Clin Cancer Res*. 2007;13:4035-4041.
165. Herbst RS, Lippman SM. Molecular signatures of lung cancer—toward personalized therapy. *N Engl J Med*. 2007;356:76-78.
166. Reid BJ. Cancer risk assessment and cancer prevention: promises and challenges. *Cancer Prev Res (Phila)*. 2008;1:229-232.

Christopher L. Corless

59 Molecular Pathology

One of the primary challenges in the clinical management of cancer patients is to establish the correct diagnosis. For over a century, the primary means of diagnosis has been tumor microscopy, an approach that can readily distinguish benign from malignant conditions, but falls short on providing the additional detail needed to guide modern therapies. As a result, a number of technologies have been developed and are now routinely employed to molecularly subtype cancers. These include the application of antibodies to detect specific proteins and other moieties within a tumor, the use of fluorescently labeled probes to identify chromosomal changes within the nucleus, and the analysis of DNA and RNA extracted from the lesion. This chapter provides an overview of these technologies, which are increasingly important in the clinical setting. Used together, they provide far more information about a cancer than the traditional microscope, providing insight into the inherent aggressiveness of the tumor and the likelihood of its response to specific therapies.

Processing Cancer Specimens for Microscopic Evaluation

Samples of cancer tissue, whether from a biopsy or a complete surgical resection, are processed to generate microscopic sections in one of two ways. The first approach is to freeze the tissue in a semiliquid mounting medium that hardens at −20° C, allowing sections to be cut inside a refrigerated cabinet (a cryostat). The tissue in the resulting sections remains unfixed and can be used for a number of types of studies. In clinical pathology laboratories, such sections are routinely used as a rapid approach (5 to 10 minutes) for assessing the presence or absence of tumor within a given sample, or to evaluate the margins of a resected malignancy. This allows the pathologist to give immediate feedback to the surgeon in the operating room.

The second approach to creating microscopic sections of tumor tissue is to "fix" it in a preservative and then embed it in paraffin for sectioning at room temperature. First developed in the 19th century, the combination of fixation and paraffin embedding yields superior morphologic detail under the microscope as compared with cryosectioning. Organic fixatives such as acetone, methanol, or ethanol leave nucleic acids largely intact, but they are not used in clinical labs because the morphology is not as good as with for maldehyde. Nearly all clinical laboratories use formaldehyde because of this advantage, even though this fixative leads to extensive crosslinking of nucleic acids as well as spontaneous deamidation of cytosines. As a result, nucleic acids recovered from formaldehyde-fixed tissue are often highly degraded. The process of paraffin embedding adds additional insult to the quality of the tissue, as it requires dehydration through organic solvents such as alcohol and xylenes, followed by exposure to molten paraffin. Lipids are completely lost, and proteins and nucleic acids are greatly altered. Nevertheless, the vast majority of cancer specimens available from clinical archives are formalin-fixed, paraffin-embedded (FFPE) tissues.

The one advantage to FFPE tissue is that it is quite stable and can be used for studies over many years to decades. Indeed, FFPE tissue samples from autopsies performed during the influenza pandemic of 1918 were used 80 years later to help molecularly subtype the virus.[1] However, proteins and nucleic acids in FFPE tissues are greatly altered, and this has a significant impact on the types of assays that can be performed on them. For example, genomic DNA from FFPE tissue is highly fragmented, with an average size ranging from 250 to 500 bp, depending on the length of fixation. Likewise, RNA is very degraded and limited to the range of 100 to 200 bp in length.

Special Stains for Evaluating Cancer Tissues

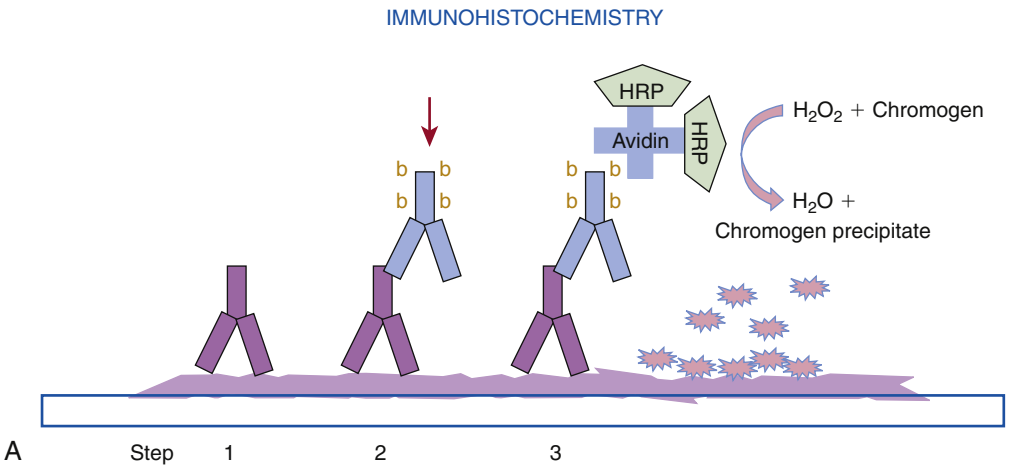
The standard histological stain for examining tissue sections on a microscope is a combination of hematoxylin, which binds nucleic acids and yields a blue color, and eosin, which

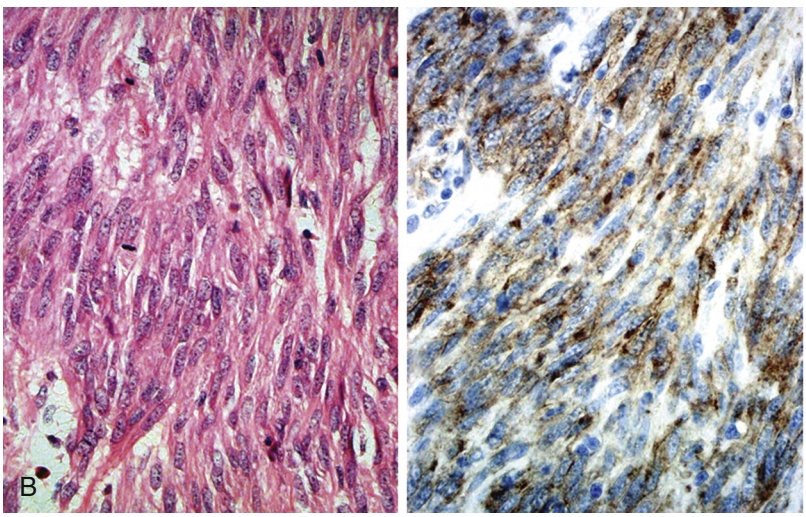
stains protein-rich materials pink to red. This "H&E" stain is routinely performed on all specimens processed in clinical pathology laboratories and can be readily interpreted by anyone trained in histopathology. Other special histochemical stains can be used to highlight fibrous tissue or deposits such as iron, copper, or amyloid. However, the most useful special stains for classifying cancer specimens are based on immunodetection of specific molecules using antibodies.

Immunohistochemistry

First developed in the late 1960s, immunohistochemistry has become a standard tool for evaluating samples of cancer tissue.[2] This approach takes advantage of the exquisite specificity of antibodies to detect particular proteins or protein modifications (phosphates, sulfates, carbohydrates, and other moieties) in tissues (Figure 59-1). Visualization of a bound antibody is through a secondary enzymatic reaction that leaves a brown, red, or black deposit visible through the microscope. In most clinical laboratories, the process is fully automated and performed in about 2 hours, making this a rapid, cost-effective tool for defining the basic molecular features of cancer samples.

Thousands of antibodies have been developed for use in immunohistochemistry, and they serve to define key characteristics of tumor cells. For example, determining whether a high-grade malignancy represents a carcinoma, melanoma, sarcoma, or lymphoma can be very challenging based on microscopic morphology, but the diagnosis is readily settled with just a few stains. Other antibodies serve as highly specific lineage markers (see Figure 59-1). For example, thyroid transcription factor 1 (TTF1) is expressed only in carcinomas originating in the thyroid and lung, CDX2 is expressed in adenocarcinomas of intestinal origin, HMB45 is a marker of melanomas, and OCT4 is specific for some types of germ cell tumors.[3-6] These types of antibodies are routinely used in combination to help confirm or exclude a suspected site





Hematoxylin & eosin stain Immunohistochemical stain for KIT

Figure 59-1 Immunohistochemistry (A) (*1*) An antibody specific for a particular protein, phosphate, sulfate, carbohydrate, or other moiety is allowed to bind to the tissue section. (*2*) A secondary "anti-antibody" is added, bearing numerous biotin tags. (*3*) A complex of avidin and horseradish peroxidase (HRP) is added. Avidin binds tightly to the biotin on the secondary antibody, while HRP reacts with a chromogen in the presence of H_2O_2, producing a visible precipitate on the tissue. **(B)** Microscopic images of a gastrointestinal stromal tumor. The brown staining in the right image represents immunohistochemical detection of the KIT tyrosine kinase (CD117) in the tumor.

of origin for a cancer in a patient with metastatic disease. Finally, antibodies have been developed to detect specific protein variants, such as EGFR variant III (deletion of exons 2 through 7) in gliomas, or BRAF harboring the V600E mutation, which is an important treatment target in melanoma and other cancers.[7,8]

Although immunohistochemistry is a mainstay in modern pathology, it does have limitations. Not all antibodies are perfectly specific; background issues and cross reactivity can affect interpretation. Further, the technique is at best only semiquantitative and can be influenced by the length of formaldehyde fixation and many other parameters. Some of these limitations can be mitigated by automation of the staining, but tumor necrosis, intraoperative ischemia, and variation in specimen processing are all factors that can complicate the reading of immunohistochemical stains.

Immunofluorescence

Because formaldehyde fixation greatly distorts or even destroys many epitopes in tissue samples, there are numerous antibodies that will not work on FFPE sections. Such antibodies may still be useful, however, on cryostat sections, which can be prepared if fresh-frozen tissue is available. In these circumstances, fluorescence rather than histochemistry is the preferred method of detection, as it has greater sensitivity and allows for several antibodies to be stained at once (using different fluorophores).[9] Immunofluorescence is routinely used in evaluating medical diseases of the kidney and skin, but is rarely employed in cancer diagnosis.

In Situ Hybridization

Expression of particular mRNAs in tumor tissue can be measured by in situ hybridization (ISH).[10] In this approach, complementary oligonucleotide probes (either DNA or RNA) bearing a chemical tag such as digoxigenin are allowed to hybridize to their target mRNA. Once hybridized, the tag is detected with a secondary antibody (e.g., anti-digoxigenin), which is linked to an enzyme that produces a visible chemical deposit on the cells. Horseradish peroxidase and alkaline phosphatase of the two most commonly used enzymes in this powerful approach, which is widely employed in cancer research. However, ISH is technically challenging and works best on mRNAs that are expressed in high abundance. For these reasons, the use of ISH in clinical laboratories is generally restricted to the assessment of kappa and lambda light-chain expression in cases of suspected myeloma, or to detect the presence of Epstein-Barr virus in cases of lymphoproliferative disease driven by this infection.

Fluorescence in Situ Hybridization (FISH)

Nucleic acid probes are used not only to detect mRNA, but to assess interphase chromosomes in tumor cells. The probes may consist of either DNA or RNA, and they vary in length from short oligonucleotides to multigenic chromosomal segments cloned into bacteria (so-called bacterial artificial chromosomes, or BACs). When directly labeled with a fluorophore, they can be detected using a microscope equipped for immunofluorescence. This approach is routinely used in clinical cytogenetic laboratories to assess gene copy number as well as gene translocations. For example, FISH is commonly used to evaluate breast carcinomas for amplification of the ERBB2 (HER2) locus and to screen for increased copies of NMYC in neuroblastoma and MET in non–small-cell lung carcinoma. Loss of tumor suppressors such as PTEN, CDKN2A, and TP53 can also be assessed by this approach.

There is a long and growing list of gene translocation events that are linked to cancer. Whether the result of intra- or interchromosomal exchanges, these translocations commonly involve genes encoding a kinase or a transcription factor. The resulting fusion genes are often the principal drivers of tumorigenesis and therefore serve as diagnostic markers and/or targets for specific therapies (e.g., kinase inhibitors). Fusion mRNAs from translocation events can be detected by highly sensitive methodologies based on polymerase chain reaction (PCR); however, these approaches can be frustrated by the fact that a particular target gene may be fused to any of more than a dozen different partner genes, requiring numerous primers to cover all possible fusion events. In contrast, FISH can detect a translocation-related break in a target gene irrespective of which partner gene has been fused to it. This is done by labeling two pools of probes with different fluorophores; for example, one pool may be labeled red and hybridizes to the 5′ end of the gene, while the other is labeled green and hybridizes to the 3′ end of the gene. If the gene is intact, the red and green signals are close together and merge into yellow, but if the gene has been split apart by a translocation event, the red and green signals become well separated (Figure 59-2). A further advantage of FISH is that only a few tumor cells are required to make a diagnosis, which is helpful in biopsies containing low tumor content.

Preparing Nucleic Acids from Cancer Specimens

Many of the assays that are used to molecularly subtype cancers require the extraction of either RNA or DNA from the tumor tissue. Two important issues are often overlooked in this regard. The first is that the nature of the tissue dictates the quality of

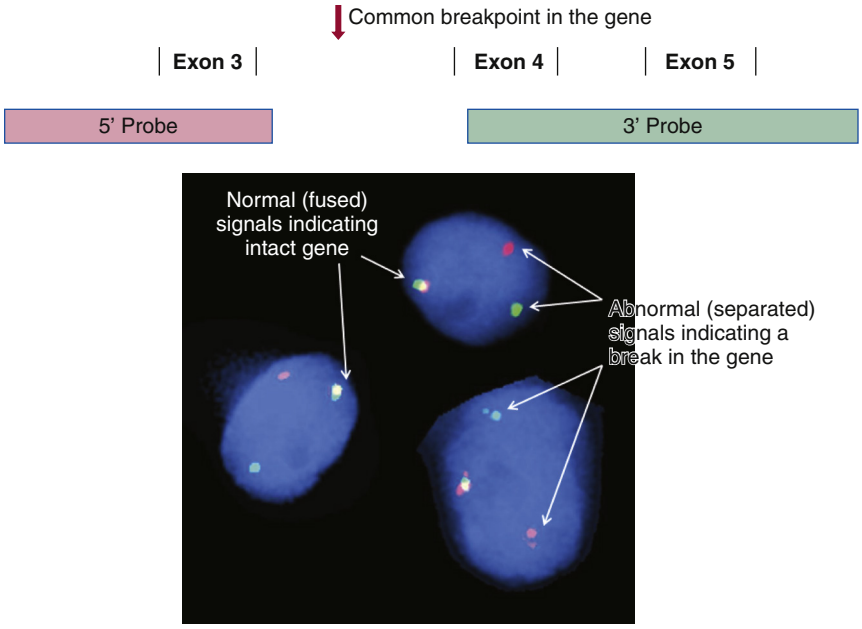


Figure 59-2 Fluorescence in situ hybridization (FISH) for identifying gene translocation events This approach is commonly used to look for evidence of gene translocations in interphase nuclei. Two probes are designed to hybridize to the 5′ and 3′ ends of a gene, flanking the region that is commonly "broken" during translocation. The probes are labeled with fluorescent tags of differing color, often creating a third color when they bind subjacently (e.g., green and red fusing into yellow). Wide separation of the probes indicates a break in the gene consistent with a translocation event. Note that this approach does not identify the partner gene in the translocation.

the available nucleic acid. Whereas fresh-frozen tissue will yield RNA or DNA that is largely intact, the nucleic acids derived from FFPE tissue are highly degraded. Thus, assays meant for use on FFPE-derived RNA or DNA must take into account the short, fragmented nature of these derivatives.

The second important issue is that tumor content and quality are highly variable from one specimen to the next, and even among different parts of the same specimen. For example, one paraffin block from a cancer resection specimen may consist of 90% tumor cells, while an adjacent block is dominated by stromal and inflammatory cells, with only 10% tumor cells being present. A third block from the same specimen may contain only necrotic tumor, as geographic necrosis is common at the center of large, high-grade malignancies. Thus, it is critical that all tumor material being considered for nucleic acid extraction first be evaluated by a qualified pathologist, who can assess whether the tumor sample is suitable for the proposed testing.

Tumor Enrichment by Macrodissection

The sensitivity of molecular diagnostic assays for mutations and other alterations in tumor DNA is dependent in part on the ratio of tumor DNA to normal cellular DNA present within a specimen. If 90% of the cells within a tissue sample are cancerous, mutation detection is straightforward, but if only 10% of the cells represent the tumor, DNA from the normal cells will effectively dilute out the mutation and make it harder to detect. For this reason, it has become standard practice to dissect out the tumor-rich areas of a specimen. This can be performed under a dissection microscope, but is more commonly done by simply scraping the tumor from unstained paraffin sections using a scalpel blade, or by taking a small core directly out of the paraffin block. Comparison with an H&E-stained section serves to guide the dissection.

Tumor Enrichment by Laser Capture Microdissection

Some tumor samples have too little tumor to allow macrodissection. In these cases, a microscope equipped with a laser can be used to isolate small clusters of tumor cells, or even single cells, from adjacent normal tissue elements. Although this is labor intensive and yields relatively little material for further testing, it can be used to salvage cases when no other tumor material from a patient is available.

Assays for Single Genes or Single Mutations

Over the past two decades, a long and growing list of genes that play a significant role in cancer has been identified. For mutations in those genes that are of particular prognostic importance or serve as important predictors of therapeutic response, a variety of assays have been developed for use in clinical laboratories. Most of these assays are focused on a single mutation or a set of mutations occurring in a single gene exon. Important factors in the design of such an assay include the amount of input DNA or RNA needed, the ease and rapidity with which the assay can be completed, and its

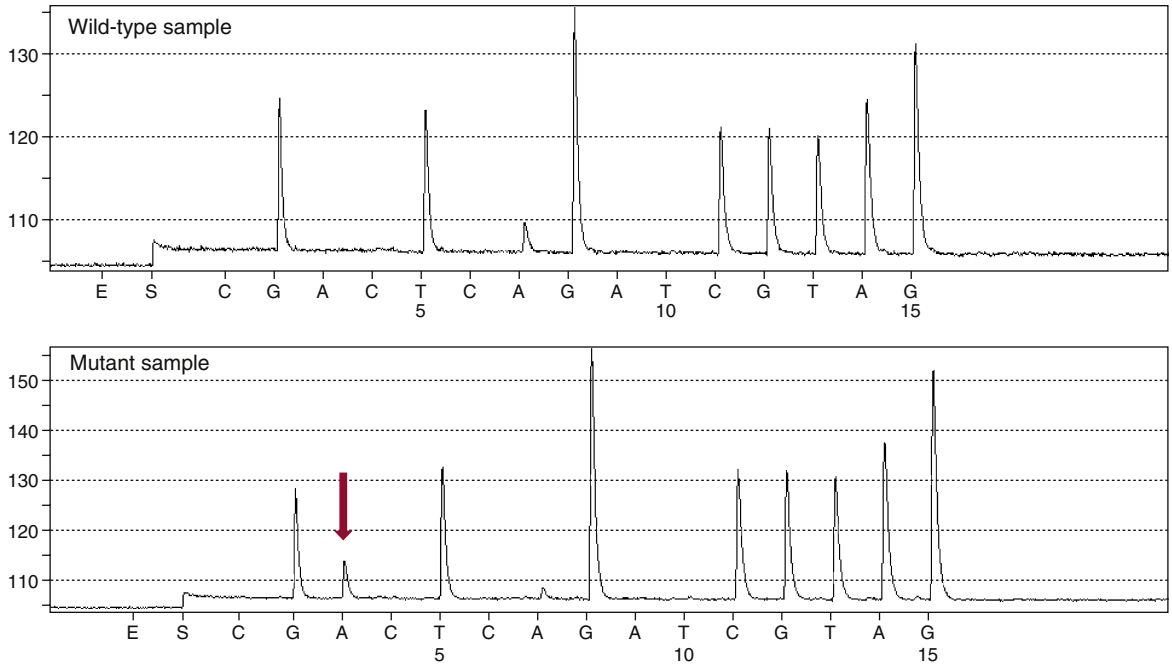


Figure 59-3 Pyrosequencing Two pyrograms are shown from tumor samples subjected to pyrosequencing for common mutations in the KRAS gene. The presence of an extra A peak in the lower panel is evidence for the presence of a mutation; the preceding G peak has a correspondingly lower signal, indicating a G→A substitution.

sensitivity and specificity. Of course, assay cost is another important factor. There are many different platforms used to support these types of assays, and each represents a significant investment for the laboratory.

Sanger Sequencing

The single most common approach to screening mutations in cancer genes is Sanger sequencing. First described by Fred Sanger and colleagues in 1976, this method mixes nonextendable, fluorescently labeled dideoxy nucleotides together with standard nucleotides to generate a set of fragments of varying length as the original template DNA is copied.[11] When separated by capillary electrophoresis, the sequence of the DNA can be interpreted from the color of the last incorporated (dideoxy) nucleotide on each successive fragment. Highly reliable and reproducible, Sanger sequencing was used to generate the first complete sequence of the human genome.[12] Nevertheless, it has several significant drawbacks. First, it can only detect mutations that are present in at least 15% to 20% of the input DNA molecules. Second, it takes approximately 24 hours to perform and is relatively labor intensive. Third, it can only cover mutations across approximately 200 bp of FFPE DNA. This means that many genes require multiple, overlapping sequencing reactions in order to ensure that a mutation can be identified, adding to the overall cost. For this reason, many laboratories rely on other approaches for screening common cancer-related mutations.

Pyrosequencing

Developed as a rapid and sensitive approach to sequencing very short regions of DNA (up to approximately 30 bp), pyrosequencing is used by many laboratories to screen for common mutations in tumor oncogenes such as KRAS, EGFR, and BRAF.[13] This technology differs from Sanger sequencing in that it does not require the synthesis and electrophoretic separation of DNA fragments. Instead, the incorporation of nucleotides during DNA synthesis is measured through a secondary reaction: each pyrophosphate released during nucleotide incorporation results in the generation of a light signal by the enzyme luciferase. As dATP, dCTP, dGTP, and dTTP are sequentially added, their incorporation is detected by the luciferase reaction, allowing the DNA sequence to be interpreted (Figure 59-3). The principal advantages of pyrosequencing are speed (sequencing can be completed in 2 hours) and sensitivity (as low as 5% mutant allele). A modification of the method is commonly used to assess DNA methylation.

High-Resolution Melting Curve Analysis

First developed in the late 1990s by Wittwer and colleagues, high-resolution melting curve analysis is now the backbone of a variety of assays designed to quickly and cost-effectively identify the presence of a mutation.[14] After the DNA of interest is amplified by standard PCR, the melting analysis

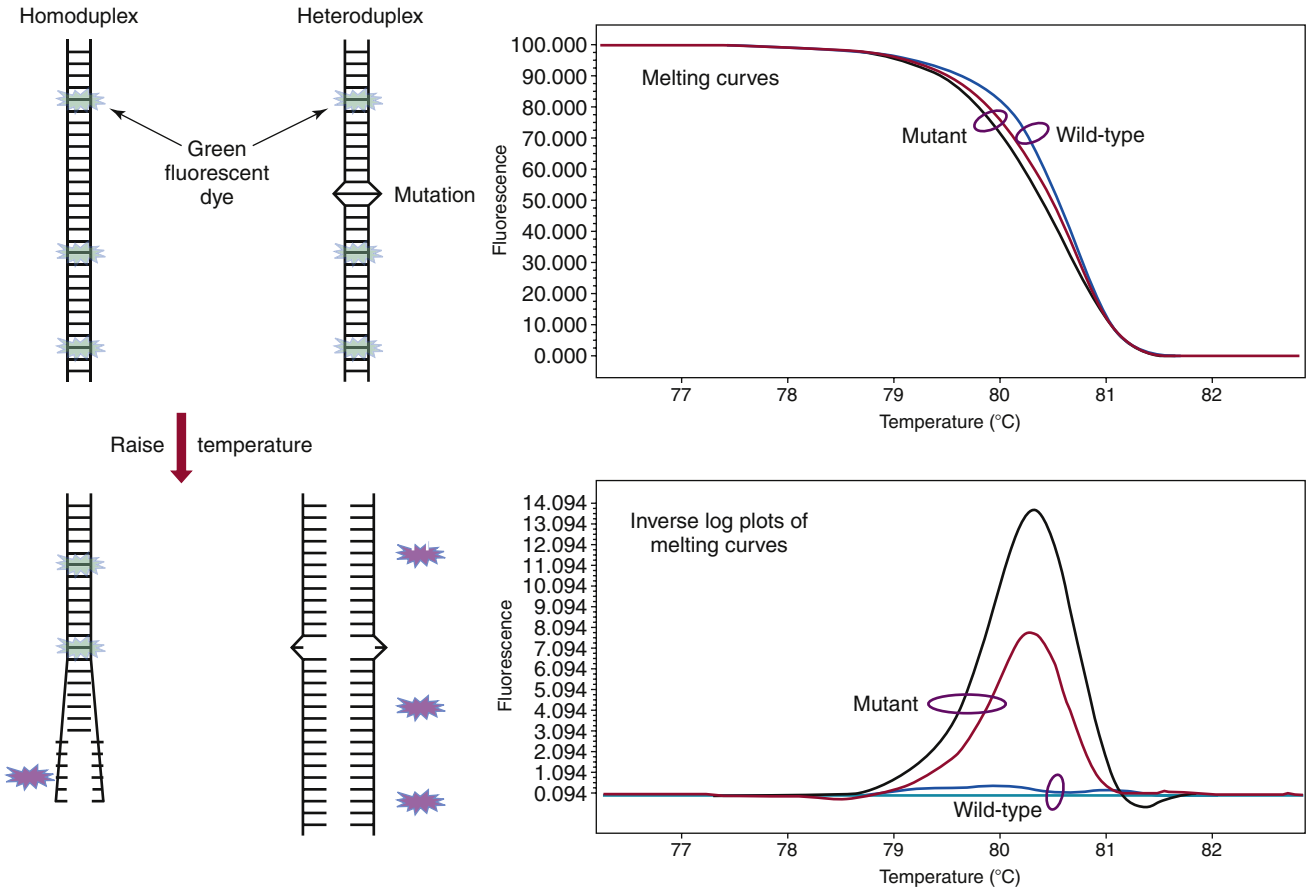


Figure 59-4 High-resolution melting curve analysis This method takes advantage of the fact that DNA heteroduplexes formed when a mutation is present have a lower melting temperature than do homoduplexes. The resulting shift in the melting curve can be detected by loss of fluorescent signal as a dye intercalated into the double-stranded DNA is released. Plotting the inverse log of the slope of the melting curve highlights the differences between samples that are wild-type and those containing a mutation. The approach can be used to detect point mutations, insertions, and deletions within amplicons of up to several hundred base pairs.

can be performed in approximately 20 minutes without the need to add reagents or switch to a different instrument. The analysis begins by melting apart the DNA strands of the final DNA product at 95° C and then allowing them to re-anneal as they cool. When a mixture of wild-type and mutant DNA is present in the sample, heteroduplexes are formed, and these can be detected by slowly ramping the temperature back up to 95° C in the presence of a fluorescent dye that binds preferentially to double-stranded DNA. As the temperature increases, the heteroduplexes unravel at a slightly lower temperature than the homoduplexes of wild-type/wild-type or mutant/mutant DNA (Figure 59-4). Thus, the presence of a mutation shifts the melting point of the overall population, and this shift is readily detected by a decrease in fluorescence as the DNA strands separate.

A number of variations of this assay type have been developed. Some will allow exact identification of a specific mutation, whereas others are optimized to detect intragenic deletions or insertions, the sequence of which requires confirmatory Sanger sequencing. In general, the sensitivity of these assays is approximately 10% mutant allele.

Allele-Specific PCR

A number of approaches have been developed that promote selective PCR amplification of a mutant allele over a wild-type allele.[15] Each depends on a primer that selectively binds to a site of mutation, the specificity being determined by the degree to which the mutant allele is favored over the wild type. Modifications to the primer, such as inclusion of peptide nucleic acids or locked nucleic acids, are generally necessary to achieve the selectivity that is optimal for clinical use.[16] The best assays have detection levels below 1%, with virtually no background contributed by false priming of the wild-type allele.

Multiplexed Approaches to Cancer Genotyping

The past decade has witnessed an explosion in the number of genes identified as playing a causal role in tumor

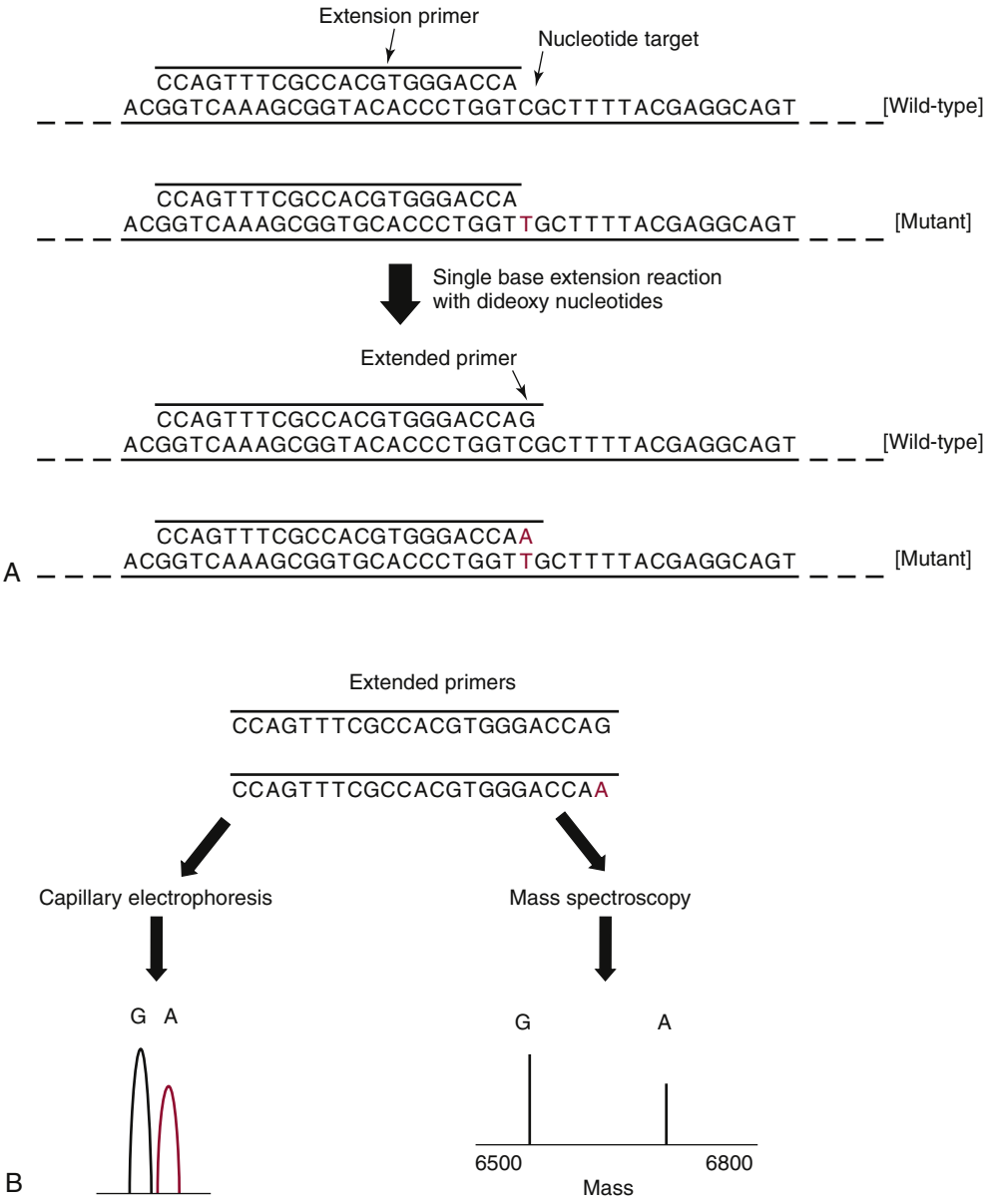


Figure 59-5 Primer extension assays These assays are used to interrogate specific "hotspot" sites of mutation occurring in oncogenes. **(A)** After an initial PCR amplification of the target sequence, an extension primer is added that sits immediately adjacent to the nucleotide of interest. **(B)** A single base extension reaction is then performed, and the products are analyzed either by capillary electrophoresis or mass spectroscopy. Both readouts are quantitative and have similar levels of sensitivity (5% to 10% mutant allele). *PCR*, Polymerase chain reaction.

development. This growing list of genes presents a challenge to clinical laboratories, which have traditionally used single-gene or single-exon approaches to genotyping. An alternative is to screen multiple genes/exons simultaneously using a multiplexed method, thereby saving on the labor and cost of performing multiple Sanger sequencing reactions. Two platforms have been developed to rapidly and cost-effectively screen for up to several hundred cancer gene mutations simultaneously. Both of these platforms—SnaPshot assays and mass spectroscopy–based assays—are based on so-called primer extension reactions.[17] In brief, each gene/exon of interest is amplified by standard PCR and then allowed to anneal to an oligonucleotide primer that sits immediately adjacent to a potential site of mutation. Dideoxy nucleotides are then added, and the primer is extended by a single base (essentially a single-base sequencing reaction), following which the product is interrogated to determine whether the added base represents wild-type DNA, a mutation, or both (Figure 59-5). The power of this approach is that the analysis can be performed in a multiplex manner, allowing many mutation sites to be screened at the same time.

SNaPshot Assays

Iafrate and colleagues were among the first to adapt this technology for screening oncogenic mutations in FFPE tumor DNA.[17] The initial PCR and subsequent primer extension reactions are multiplexed to the level of 5 to 10 reactions per tube; extended primers are then separated by capillary

electrophoresis and visualized via fluorescent tags present on the incorporated dideoxy nucleotides. Primers interrogating approximately 10 different mutation sites can be analyzed simultaneously on a single capillary. By running several multiplexes, more than 100 mutations can be screened across one or two dozen genes in approximately 24 hours. SNaPshot assays have excellent sensitivity (5% mutant allele) and are cost effective, making them popular in clinical diagnostic laboratories.

Mass Spectroscopy–Based Assays

These assays follow the same approach as SNaPshot assays, multiplexing PCR and primer extension reactions to the level of 10 to 30 per tube, but the readout on the extended primers is performed by mass spectroscopy instead of capillary electrophoresis. Although the sensitivity of the mass spectrometer is a bit lower (10% mutant allele), the throughput is significantly greater, as the instrument can read out 384 multiplexes (more than 4000 mutation sites) in 90 minutes.[18,19]

Next-Generation DNA Sequencing

The development of so-called next-generation sequencing has transformed the study of cancer by making it possible, and affordable, to generate large amounts of sequencing data in a reasonably short period of time. There are a number of different "next-gen" platforms in use, but they all share a common approach, which is to generate sequence from single molecules of genomic DNA or cDNA in a massively parallel fashion—hence the alternative name *massively parallel sequencing* (MPSS). This differs from traditional Sanger sequencing, which generates data from a population of DNA molecules that are all co-amplified during the initial PCR step.

How do MPSS systems determine the sequence of single fragments of DNA? A number of approaches are being used, and more are in development. For example, a platform developed by Pacific Biosciences is capable of visualizing individual incorporation events as nucleotides are added to a single molecule of template DNA. This extraordinary feat allows the sequencing of very long stretches of DNA (up to 10,000 bp) but is subject to relatively high error rates. Other companies are developing nanopores that will allow a single DNA molecule to pass through under an electric field. Sequence is then interpreted as the DNA molecule (either intact or as serially degraded single nucleotides) moves through the narrow channel or pore. These technologies hold the promise of extremely high throughput, but they are still in early development.

Most next-gen sequencing is currently being performed on platforms that determine the sequence from clones rather than single molecules of DNA. Clones generate more signal during the actual sequencing reaction and are therefore easier to work with. There are two common methods for generating libraries of clones from original DNA molecules. One is to seed the single DNA molecules across the surface of a glass slide and then PCR-amplify each in its place, creating microcolonies of identical DNAs.[20] These colonies, or clones, provide the substrate for subsequent sequencing reactions. The other approach is to mix a single DNA molecule together with a small sphere, or particle, within a tiny droplet of water suspended in oil (an emulsion).[21] Also included in the droplet are DNA polymerase and dNTPs, leading to amplification of the DNA across the surface of the sphere as the emulsion is subjected to PCR thermocycling. Both approaches generate a library consisting of thousands to millions of clonal DNA fragments, which are then sequenced all at the same time in a parallel fashion.

Depending on how the library of clones is generated, there may be anywhere from a few dozen to several thousand clones that represent copies of a particular segment of the original DNA. The resulting sequence from each clone is called a *read*. The sensitivity of MPSS for sequence alterations is directly determined by the number of reads that is generated for each segment of the original DNA. When sequencing the whole genome, 30 reads (or 30-fold coverage) is considered a minimum amount of data. For studies focused on the coding regions of the genome (the *exome*, representing approximately 1.8% of the total genome), read depths are generally greater than 100, whereas assays that are limited to a few hundred genes or less will often have read depths greater than 500, supporting mutation sensitivities below 5% mutant allele.[22]

An important consideration when evaluating MPSS data is the quality of the sequence reads. This can be influenced by many factors, including the source of the DNA or RNA/cDNA (e.g., FFPE versus fresh-frozen), the length of the reads, and whether the sequencing was performed in just one or both directions on the same piece of DNA. Another factor is the level of noise in MPSS datasets. Random errors occur during the initial clonal amplifications, as well as during the subsequent sequencing steps, creating variations in the sequence that must be distinguished from real changes. Finally, when MPSS is used to search for possible mutations in tumor DNA, it is best to compare the results with the sequence of germline DNA from the same individual, thereby defining which alterations are somatic. Without this comparison to the germline, it can be difficult to distinguish mutations from natural polymorphisms, which are abundant in all genomes.

Next-Generation Sequencing Based on Fluorescence Detection

There are several next-generation sequencing platforms that are based on the detection of fluorescence events during sequencing. The 454 system (Roche) is based on combination of emulsion PCR and pyrosequencing, using the same chemistry as described under single-gene assays.[23,24] The SOLiD system (Life Technologies) performs sequencing on glass slides by measuring the binding and ligation of short, fluorescently tagged oligonucleotides as they match up to the template DNA.[20] The most widely used systems, from Illumina Corp., also perform sequencing on glass slides, but measure the incorporation of individual fluorescently labeled nucleotides, similar to those used in Sanger sequencing.[25] The throughput on the Illumina systems has been expanded to the point where several whole-genome sequences can be generated simultaneously, while the cost per sample has decreased dramatically.

Next-Generation Sequencing Based on Semiconductors

One drawback to the fluorescence-based next-generation sequencing platforms is that they generate large numbers of very high-resolution image files, requiring terabytes of storage and considerable computing power to analyze. An alternative to the use of fluorescence for sequencing has been developed by Ion Torrent (Life Technologies). Their platform looks for the pH change generated as a nucleotide is incorporated into a nascent strand of DNA (a hydrogen ion is released).[26] Sequencing is performed on modified semiconductor chips containing between 1.2 million and 1.1 billion nanowells, each serving as a micro pH meter (Figure 59-6). Nanospheres that have been clonally loaded with DNA by emulsion PCR are seeded into the nanowells on the chip, and sequencing commences as nucleotides are serially flowed across the chip surface. Because pH changes are converted directly into electrical signals, this approach is very

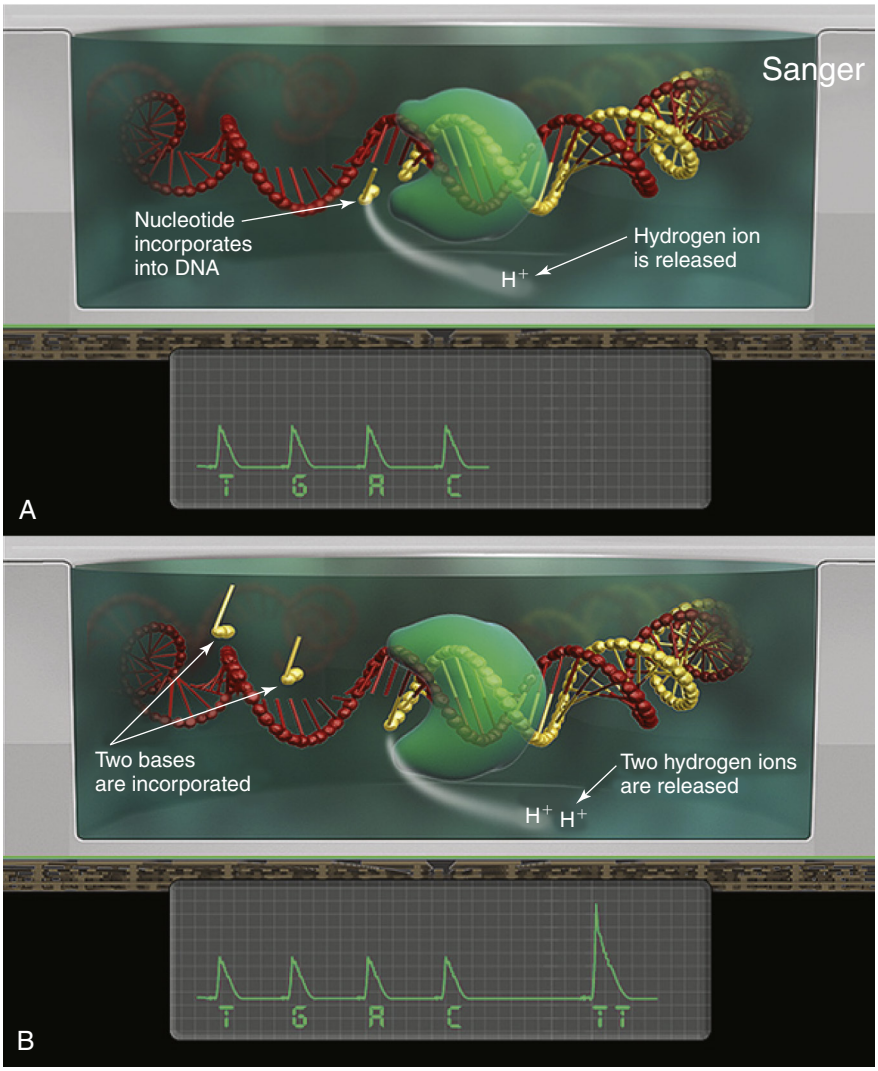


Figure 59-6 Semiconductor-based sequencing This method sequences DNA that has been clonally amplified onto nanospheres (0.5 to 3 μm) by measuring a drop in pH when hydrogen ions are released during nucleotide incorporation. **(A)** The nanospheres are distributed across a series of microwells, numbering from 1 to 660 million on a chip, each serving as an individual micro-pH meter. **(B)** As nucleotides are serially diffused across the chip surface, they are incorporated onto the nanosphere template DNA, and the resulting pH changes are interpreted to derive the template sequence.

fast and generates sequence files that are smaller than those from fluorescence-based platforms.

Future Directions

The diagnosis of cancer has been, and will continue to be, a process that depends heavily on microscopic examination of the tissue. Identifying areas of neoplasia, determining their relationship with surrounding normal tissue elements, and assessing the extent of the disease are all key elements in defining the characteristics of a malignancy. However, the field of pathology is quickly moving from routine histological assessments to increasingly sophisticated assays for molecular markers that have prognostic and/or predictive importance. The introduction of next-generation sequencing is serving to uncover the true diversity of cancers as well as to define recurring mutations that can be targeted with new therapies. Such genomic-level analyses will continue to have an impact for many years. Some have even predicted that sequencing will make microscopy moot; however, this view ignores the growing importance of assessing the relationship of cancer cells to surrounding stromal cells and to immune cells—whose influence is critical to cancer cell survival. Thus, imaging will also play a critical role in coming years, as new in situ approaches to measuring molecular events are developed and used to help further the field.

Key References

4. Werling RW, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol*. 2003;27:303-310.
5. Jones TD, Ulbright TM, Eble JN, Baldridge LA, Cheng L. OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. *Am J Surg Pathol*. 2004;28:935-940.
7. Wikstrand CJ, Hale LP, Batra SK, et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res*. 1995;55:3140-3148.
8. Capper D, Preusser M, Habel A, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. *Acta Neuropathol*. 2011;122:11-19.
11. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol*. 1975;94:441-448.
12. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860-921.
16. Strerath M, Detmer I, Gaster J, Marx A. Modified oligonucleotides as tools for allele-specific amplification. *Methods Mol Biol*. 2007;402:317-328.
17. Su Z, Dias-Santagata D, Duke M, et al. A platform for rapid detection of multiple oncogenic mutations with relevance to targeted therapy in non-small-cell lung cancer. *J Mol Diagn*. 2011;13:74-84.
18. MacConaill LE, Campbell CD, Kehoe SM, et al. Profiling critical cancer gene mutations in clinical tumor samples. *PLoS One*. 2009;4:e7887.
19. Beadling C, Heinrich MC, Warrick A, et al. Multiplex mutation screening by mass spectrometry evaluation of 820 cases from a personalized cancer medicine registry. *J Mol Diagn*. 2011;13:504-513.
20. Shendure J, Porreca GJ, Reppas NB, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*. 2005;309:1728-1732.
21. Nakano M, Komatsu J, Matsuura S, Takashima K, Katsura S, Mizuno A. Single-molecule PCR using water-in-oil emulsion. *J Biotechnol*. 2003;102:117-124.
22. Wagle N, Berger MF, Davis MJ, et al. High-throughput detection of actionable genomic alterations in clinical tumor samples by targeted, massively parallel sequencing. *Cancer Discov*. 2012;2:82-93.
23. Margulies M, Egholm M, Altman WE, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005;437:376-380.
24. Wheeler DA, Srinivasan M, Egholm M, et al. The complete genome of an individual by massively parallel DNA sequencing. *Nature*. 2008;452:872-876.
25. Hillier LW, Marth GT, Quinlan AR, et al. Whole-genome sequencing and variant discovery in *C. elegans*. *Nat Methods*. 2008;5:183-188.
26. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. *Nature*. 2011;475:348-352.

References

1. Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A*. 1999;96:1651-1656.
2. Archer FL, Kao VC. Immunohistochemical identification of actomyosin in myoepithelium of human tissues. *Lab Invest*. 1968;18:669-674.
3. Fabbro D, Di Loreto C, Stamerra O, Beltrami CA, Lonigro R, Damante G. TTF-1 gene expression in human lung tumours. *Eur J Cancer*. 1996;32A:512-517.
4. Werling RW, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol*. 2003;27:303-310.
5. Jones TD, Ulbright TM, Eble JN, Baldridge LA, Cheng L. OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. *Am J Surg Pathol*. 2004;28:935-940.
6. Mottolese M, Venturo I, Benevolo M, et al. Immunocytochemical diagnosis of amelanotic metastatic melanoma using monoclonal antibodies HMB-45 and Ep1-3. *Melanoma Res*. 1994;4:53-58.
7. Wikstrand CJ, Hale LP, Batra SK, et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res*. 1995;55:3140-3148.
8. Capper D, Preusser M, Habel A, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. *Acta Neuropathol*. 2011;122:11-19.
9. Mellors RC. The application of labeled antibody technics in studying cell antigens. *Cancer Res*. 1968;28:1372-1381.
10. Varndell IM, Polak JM, Sikri KL, Minth CD, Bloom SR, Dixon JE. Visualisation of messenger RNA directing peptide synthesis by in situ hybridisation using a novel single-stranded cDNA probe. Potential for the investigation of gene expression and endocrine cell activity. *Histochemistry*. 1984;81:597-601.
11. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol*. 1975;94:441-448.
12. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860-921.
13. Ronaghi M, Uhlén M, Nyrén P. A sequencing method based on real-time pyrophosphate. *Science*. 1998;281:363, 365.
14. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*. 1997;245:154-160.
15. Sommer SS, Cassady JD, Sobell JL, Bottema CD. A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of phenylketonuria. *Mayo Clin Proc*. 1989;64:1361-1372.
16. Strerath M, Detmer I, Gaster J, Marx A. Modified oligonucleotides as tools for allele-specific amplification. *Methods Mol Biol*. 2007;402:317-328.
17. Su Z, Dias-Santagata D, Duke M, et al. A platform for rapid detection of multiple oncogenic mutations with relevance to targeted therapy in non-small-cell lung cancer. *J Mol Diagn*. 2011;13:74-84.
18. MacConaill LE, Campbell CD, Kehoe SM, et al. Profiling critical cancer gene mutations in clinical tumor samples. *PLoS One*. 2009;4:e7887.
19. Beadling C, Heinrich MC, Warrick A, et al. Multiplex mutation screening by mass spectrometry evaluation of 820 cases from a personalized cancer medicine registry. *J Mol Diagn*. 2011;13:504-513.
20. Shendure J, Porreca GJ, Reppas NB, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*. 2005;309:1728-1732.
21. Nakano M, Komatsu J, Matsuura S, Takashima K, Katsura S, Mizuno A. Single-molecule PCR using water-in-oil emulsion. *J Biotechnol*. 2003;102:117-124.
22. Wagle N, Berger MF, Davis MJ, et al. High-throughput detection of actionable genomic alterations in clinical tumor samples by targeted, massively parallel sequencing. *Cancer Discov*. 2012;2:82-93.
23. Margulies M, Egholm M, Altman WE, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005;437:376-380.
24. Wheeler DA, Srinivasan M, Egholm M, et al. The complete genome of an individual by massively parallel DNA sequencing. *Nature*. 2008;452:872-876.
25. Hillier LW, Marth GT, Quinlan AR, et al. Whole-genome sequencing and variant discovery in *C. elegans*. *Nat Methods*. 2008;5:183-188.
26. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. *Nature*. 2011;475:348-352.

Index

Page numbers followed by *f* indicate figures; *t*, tables.

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z